ABSTRACT

Title of dissertation: EFFICIENCY AND ECOLOGICAL RISKS OF REDUCING SOIL PH DURING *THLASPI CAERULESCENS* PHYTOEXTRACTION OF CADMIUM AND ZINC

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The major aims of this research were to determine whether reducing soil pH can enhance phytoextraction and to examine the ecological risks of reducing pH. Two soils differing in Cd and Zn concentrations were used and adjusted to 5 or 6 different pH levels ranging from 7.27 to 4.74 and seeded with a hyperaccumulator of Cd and Zn, *Thlaspi caerulescens*. Plants were harvested after six months, the pH were restored to above 6.5, incubated for 6 months. Soils were analyzed for biological activities and microbial population changes after both pH adjustments.

Reducing pH significantly ($p=0.05$) enhanced plant metal uptake. For the high metal soil, plant grew best at the lowest pH treatment (4.74) and the highest metal concentration

was at the second lowest pH treatment (5.27). For the low metal soil, due to low pH induced Al and Mn toxicity, plant growth and metal uptake were highest at the intermediate pH level (6.07). Metal sequential extraction results further verified that reducing pH redistributed Cd and Zn among five fractions. The most soluble metal form (F1) was greatly increased. In addition, *T. caerulescens* was able to differentially utilize Cd in all 5 fractions while it could only access Zn from the F1 and F2 pools.

Reducing soil pH significantly reduced a number of soil biological activities and shifted the community structure at different levels. Generally, soil biological activities were more sensitive than soil microbial populations to pH change. Good indicators of soil pH status were acid phosphatase activity, alkaline phosphatase activity, acid to alkaline phosphatase activity ratio, arylsulphatase, nitrification potential, soil microbial biomass C and N, and population of rhizobium. After raising pH to > 6.5, negatively impacted soil parameters were partially restored to original levels. Soil biological activities showed lower recovery than soil microbial populations. The threshold pHs were 6.1 and 5.3 for low and high metal soils, respectively. Above this value, most soil biological activities and all microbial populations returned to background levels within a short period.

EFFICIENCY AND ECOLOGICAL RISKS OF REDUCING SOIL PH DURING *THLASPI CAERULESCENS* PHYTOEXTRACTION OF CADMIUM AND ZINC

by

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Dissertation submitted to the Faculty of the Graduate School of
the University of Maryland at College Park in partial
fulfillment of the requirement for the degree of
Doctor of Philosophy
2004

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ACKNOWLEDGMENTS

First of all, I am deeply grateful to Dr. J. Scott Angle, for his advice and unreserved support whenever I needed throughout this research. Dr. Angle accepted me and gave me this great opportunity to work on phytoremediation and soil microbial ecology. I want to thank Dr. Rufus Chaney, for his encouragement, guidance, for being there whenever I had questions or needed help. Dr. Chaney's enthusiasm towards science and his pursuit to do "good science" helped to shape my own scientific value.

Dr. Marla McIntosh deserves a special thank you for your help, for being the next door neighbor to my lab and the tolerance of my interruption whenever I had questions for you. I would like to thank Dr. Greg McCarty, for opening his lab and assisting in doing several analyses for this research. I appreciate Dr. Allen Davis, for his time and expertise.

I am indebted to Dr. Thierry Delorme, for his inspiration through our numerous discussions and his assistance; Dr. Carrie Green, for her help, patience, and encouragement; my fellow graduate students Salil Chanroj, Lan Zhang, and Carol Gordon, for their help and moral support. My gratitude also goes to Mattew Fagan, Chenping Xu, Xiaoyu Tang, and Lan Su, for their excellent lab assistance.

My family offered loving support and steadfast encouragement even though they are far away. Their love is the strength that let me go on despite all difficulty and discouragement. Finally I am indebted to someone for whom my love knows no limits: my fiancé Tang, who has given me tremendous support and comfort, who spent tremendous amount of time helping me out in the lab, who shared my ups and downs

throughout the process and tolerated my bad mood whenever something went wrong in the experiment with a forgiving and loving heart.

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LIST OF ABBREVIATIONS

A	actinomycetes
AcP	acid phosphatase
Al	aluminum
AlP	alkaline phosphatase
As	arsenic
ATP	adenosine triphosphate
Br2a	bacteria using r2a medium
Brim	bacteria using rim medium
C	carbon
Ca	calcium
Cd	cadmium
CFU	colony forming unit
cm	centimeter
Cu	copper
DNA	deoxyribonucleic acid
F	fungi
F1	metal extraction fraction one
F2	metal extraction fraction two
F3	metal extraction fraction three
F4	metal extraction fraction four
F5	metal extraction fraction five
Fe	iron
g	gram
h	hour(s)
Hg	mercury
kg	kilogram
K	potassium
LSD	least significant difference
MBC	microbial biomass carbon
MBN	microbial biomass nitrogen
mg	milligram
Mg	magnesium
min	minute(s)
Mn	manganese
MPN	most probable number
μg	microgram
N	nitrogen
NaOAc	sodium acetate
NaOCl	sodium hypochlorite
NIST	National Institute of Standards and Technology
ns	not significant
P	phosphorous

PNP	p-nitrophenol
S	sulfur
SD	standard deviation
Se	selenium
SEP	sequential extraction procedure
$Sr(NO_3)_2$	strontium nitrate
wk	week
wt	weight
Zn	zinc

CHAPTER 1 INTRODUCTION

1.1 RESEARCH JUSTIFICATION AND RATIONALE

Successful phytoextraction relies on appropriate soil and plant management practices to attain high yields and high metal concentrations in the plant biomass. Among the diverse strategies to enhance phytoextraction, pH adjustment has been received the most attention because heavy metals' bioavailability is largely controlled by soil pH. However, studies on pH effects on *T. caerulescens* hyperaccumulation are scarce. The causal relationship between soil pH and *T. caerulescens* Zn and Cd accumulation is obscure. For phytoextraction to be successful and viable in environmental remediation, strategies that can optimize plant uptake must be identified. The uncertainty of whether adjusting soil pH is an efficient way to enhance *T. caerulescens* hyperaccumulation must by clarified.

pH, as a master variable in the soil environment, is a key factor in controlling soil ecological conditions. Reducing pH will affect many soil ecological characteristics. The ultimate goal of remediation of any kind is to regenerate a healthy soil ecosystem. If during the process, however, the soil health is further reduced, it violates the remediation principal and phytoremediation will never be widely adopted. Therefore, prior to any real world remediation, it is important to determine to what extent the adverse impact would be and whether this affect is "acceptable"? However, no such ecological risk assessment work currently exists. Furthermore, reducing soil pH in metal-rich soils may have added ecotoxicity due to increased bioavailable metal concentrations. To what extent this will contribute to the negative impacts on soil microbial populations in addition to the low pH

effect is unknown. Research is therefore required to provide knowledge in understanding such important issues regarding phytoextraction.

1.2 OBJECTIVES

The specific objectives of this study were to:

- Examine mobility and re-distribution of five sequential extraction fractions of Cd and Zn under different pH adjustments and identify the metal pools that can easily become labile under low pH.
- Determine the effect of reducing pH on *T. caerulescens* growth and hyperaccumulation of Cd and Zn and identify the optimum pH to obtain maximum plant biomass and the highest metal extraction.
- Investigate the impact of lowering soil pH in metal-contaminated soils on soil microbial ecosystems, both from soil biological activities and microbial community structure.
- Assess the ecologic risks of reducing pH in metal-rich soils on soil microbial ecosystems, i.e., examine whether, or to what degree, the negatively impacted microbial properties and altered community structure return to “normal” by increasing soil pH once phytoextraction is complete.

1.3 OVERVIEW OF THE THESIS

This thesis contains seven chapters.

Chapter 1 is a brief introduction to the present research and thesis constructure.

Chapter 2 provides a general literature review on various aspects that are pertinent to the present research.

Chapter 3 examines the effect of reducing pH on *T. caerulescens* hyperaccumulation and metal distribution among 5 sequential extraction fractions.

Chapter 4 investigates the impact of reducing pH on soil biological activities. Parameters investigated were acid phosphatase, alkaline phosphatase, arylsulfatase activities, soil nitrification potential, and basal respiration.

Chapter 5 examines soil microbial community structure changes under reduced pH by measuring the number of soil viable bacteria, actinomycetes, and fungi; soil microbial biomass carbon and nitrogen; population size of soil *R. leguminosarum* bv. *trifolii.*

The ecological risks of reducing pH are discussed in chapter 6 by comparing the changes of a number of parameters both under acidification and neutralization treatments.

Finally, an overall conclusion and implications of this work are presented in chapter 7.

CHAPTER 2 LITERUATURE REVIEW

2.1 ZINC AND CADMIUM

2.1.1 Properties and occurrences

Heavy metals are defined as a group of metallic elements with a density > 5~6 g cm^{-3} or an atomic number > 20 (Davis, 1980). According to this definition, both Zn and Cd are heavy metals. Zn has an atomic number of 30, and a configuration of $3d^{10}4s^2$. Cd, has an atomic number of 48, and a configuration of $4d^{10}5s^2$. They both belong to the IIB group in the periodic table and both easily form divalent cations. They share many similarities in chemical properties except that Cd has more polarizability due to the extra layer of d-orbital electrons interposing between the core and the valence electrons (Phipps, 1976).

Both natural weathering of rock bed and anthropogenic sources result in accumulation of heavy metals in soil (Ross, 1994; Campbell et al., 1983). Different parent materials contain differing concentrations of metal elements. Rock phosphate Cd concentrations range from 1 to 90 mg kg^{-1} (Baechle and Wolstein, 1984). The background levels of total Zn and Cd in most soils are usually <100 mg kg^{-1} and <1 mg kg^{-1}, respectively (Adriano, 1986).

Zn and Cd are used in alloys, anti-corrosion coatings on metal products, tires, paints, batteries, and many other products (Adriano, 1986). Increased global consumption has caused widespread release of these metals into biosphere (Nriagu, 1979). Agricultural usage of metal-containing fertilizers, pesticides, land application of sewage sludges and municipal wastes, smelters and the mining industry are major anthropogenic sources of

metal contamination (Chaney, 1993; Mortvedt 1987; Merry et al., 1983; Purves, 1977; Brookes and McGrath, 1984; Takijuma and Katsumi, 1973).

2.1.2 Toxicity and environmental impact

While plants have no known Cd requirement, Zn is an essential micronutrient for plant growth and performs important functions in cell metabolism (Marschner, 1986). Zn and some other essential metal ions can stabilize and activate many proteins. About one-third of all enzymes require metal ions as co-factors. De-activation occurs when a toxic metal ion replaces the natural required ion in an enzyme active center through competition (Martin, 1986). High levels of available Zn and Cd can cause severe phytotoxicity. Symptoms include chlorosis, growth reduction, and even plant death.

Normal Zn concentrations in plant dry tissue range from 25 to 150 mg kg^{-1} (Jones, 1991) and Cd levels are usually <1 mg kg^{-1} (Page et al., 1981). The typical values in leaf dry matter for Zn and Cd phytotoxicity are around 500 and 50 mg kg^{-1}, respectively (Chaney, 1993; Bingham, 1979). Severe phytotoxicity may further cause decreased genetic diversity, increased soil erosion problem, and ecosystem devastation (Chaney et al., 2000).

Elevated Zn and Cd adversely affect soil microbial ecosystems and disrupt soil functions. The documented adverse effects include decreased total soil microbial biomass (Brookes and McGrath, 1984; Chander and Brookes, 1991; Chander and Brookes, 1993), reduced soil basal respiration or/and substrate induced respiration (Speir et al., 1999; Doelman and Haanstra, 1984), inhibited soil enzyme activities (Stuczynski et al., 2003; Kuperman and Carreiro, 1997; Al-Khafaji and Tabatabai, 1979), shifted microbial

community structure (Ellis et al., 2001; Kelly et al, 1999; Frostegård et al., 1993, 1996; Barkay et al, 1985; Bååth et al, 1998), decreased catabolic functional diversity (Banerjee et al, 1997; Knight et al, 1997 b), reduced fungal populations (Nordgren et al, 1983, 1985) and delayed mycorrhizal infection of clover (Koomen and McGrath, 1990). There are also a number of studies about the impact on N_2-fixation especially on leguminous symbiotic N_2-fixation. The observed influences include decreased population size of these organisms (Chaudri et al., 1992, 1993, 2000 a, 2000 b; Giller et al., 1993; Ibekwe et al, 1996, 1998), decreased genetic diversity (Giller et al., 1989; Hirsch et al., 1993), delayed nodulation (El-Kenawy et al. 1997; Ibekwe et al, 1996, 1998), ineffective nodulation (Chaudri et al., 1992; McGrath et al., 1995), and decreased fraction of nitrogen in clover derived from fixation (Broos, et al., 2004).

The severity of heavy metal toxicity to soil microorganisms is greatly affected by soil pH. A study reported that when pH was maintained at 6.0 or above, heavy metals had no effect on either nodulation or nitrogen fixation. However, reduced nodulation and ineffective symbiosis were observed under low pH at the same metal treatments (Ibekwe et al, 1995). Similarly, heavy metal content alone was not found to be related with mycorrhizal infection; however, high metal content caused extreme pH sensitivity of mycorrhizal infection. High metal content plus low pH greatly decreased mycorrhizal infection (Angle and Heckman, 1986).

It is important to note that there is enormous variability associated with metal toxicity to soil microbial processes and populations. In a review paper, Giller et al (1998) wrote, “the highest metal concentration in the soil where no effect was found (HNOEC) and the lowest metal concentration where an effect was found (LOEC)…varied between

studies by 100 to 1000-fold for individual metals and overlapped to a great extent". These authors summarized two factors which may contribute to the discrepancies between studies "(1) factors which modify the toxicity of the metals and (2) differences in sensitivity of the microorganism(s) or microbial process(es)" (Giller et al, 1998). Specifically, one important modification factor is metal source. Sludge-borne metals may have quite different physicochemical toxicity than metal salts. Microbial responses are also complicated by nutrients and organic matter contained in sludge (Angle et al, 1993). Therefore it is not surprising to find that application of metal-containing sewage sludge enhanced the growth of rhizobia (Madariaga and Angle, 1992).

If Zn and Cd contaminated food or drinking water enters the food chain, human health is threatened. This is the ultimate concern from an anthropogenic point of view. The well-publicized case of the so-called *Itai-Itai* disease outbreak in the Jintsv River basin of Japan was caused by Cd-bearing wastewater discharged into the river from an upstream mining company (Laws, 1993). Research has shown that Zn can inhibit plant uptake of Cd provided that Zn to Cd ratio of the contaminated soil is kept at 50-200. In most Cd and Zn contaminated soils, this ratio is generally to be found around 1:100. Most crops proportionally take up Cd and Zn and keep the Cd to Zn concentration ratio in their tissue similar to levels found in soil. As long as consumers maintain a balanced diet which contains adequate Fe and Zn, less Cd will be absorbed into the body (Chaney et al, 2001). However, rice and tobacco are two exceptions. The anaerobic condition under which rice is grown prevents uptake of Zn. Rice is remarkably low in Zn and Fe compared to other crops. Long term unbalanced diet results in kidney and liver disease (Chaney and Ryan, 1994). Although Cd contaminated rice fields are most noticeably

found in eastern Asia, these types of incidents have by no means been confined to Asia. The Cd health threat is a world wide problem. In the USA, about 0.005% of the population is possibly under the risk of Cd contamination (Ryan and Chaney, 1995).

2.1.3 Forms and bioavailability in soils

Both Zn and Cd are present in many different forms in soil. Information about the physicochemical forms of Zn and Cd is needed for understanding metals' geochemical distribution, mobility, and biological availability. Sequential extraction procedures use a series of chemical reagents with increasing capacity to extract metals in different phases (Tessier et al, 1979; Lo and Yang, 1998; Luoma, 1981). Despite much controversy, sequential extraction procedures remain a useful and important technique in understanding metal forms in soil (Kim and Fergusson, 1991; Bunzl et al, 1999; Miller et al, 1986). With increasing awareness that total metal concentration has little association with phytotoxicity, it is important to quantify the various fractions of metals present in contaminated sites. A complete sequential extraction usually partitions Zn and Cd into five operationally defined forms: 1) soluble-exchangeable, comprised of free metal ions and soluble complexes, usually extracted with dilute salt solutions, 2) specifically sorbed-carbonate bound, typically extracted by 1.0 M sodium acetate, 3) oxidizable, metals primarily complexed with Fe, Mn oxides, extracted by some common reducing agents, 4) reducible, metals complexed with organic matter, extracted by H_2O_2 or NaOCl oxidizing agents, and 5) residual metals held in the primary mineral matrix, usually extracted with strong acid (Ahnstrom and Parker, 1999; Aualiitia and Pickering, 1987; Hall et al, 1996; Hickey and Kittrick, 1984; Kim and Fergusson, 1991; Shuman, 1982; Shuman, 1983).

There are numerous chemical processes in soils that influence metal solubility. pH is a key factor in determining equilibrium conditions and metal solubility. For example, heavy metals can be retained by the permanent charge sites of layer silicate clays through non-specific electrostatic forces or specific chemisorption. The irreversibility and the specificity are increased at higher pH (Farrah and Pickering, 1977; Tiller et al., 1979, 1984; McBride, 1989). Two-dimensional surface adsorption of metals by oxides, hydroxides, and amorphous alluminosillicates are partially or completely reversible by pH change (Anderson, et al., 2002; Li et al., 2001; Schwarz et al, 1999 b). Redox processes also affect metal solubility. Heavy metals are less soluble in their higher oxidation states. pH through its relationship with pe indirectly affects the soil redox status. The effects of organic matter in affecting metal solubility also depend on pH. At low pH values, organic matter adsorbs metals through an ion exchange process between H^+ and metal ions on acidic functional groups or direct coordination with functional groups. Higher pH, however, promotes the dissolution of soil organic matter and formation of soluble metal-organic complexes (Herms and Brümmer, 1982; Zachara et al, 1992; Xu et al, 1989).

At low concentrations, Zn and Cd solubility is believed to be regulated by sorption behavior through surface complexation while at high concentration levels, dissolution-precipitation equilibrium is more important (Hayes and Traina, 1998). Most soils requiring remediation fall into the latter group.

2.2 PHYTOEXTRACTION OF ZINC AND CADMIUM FROM SOILS

2.2.1 Introduction of phytoextraction and *Thlaspi caerulescens*

Phytoremediation is defined as the use of plants to remove, contain, or render harmless contaminants in soils. Possible mechanisms may include: extraction, volatilization, rhizofiltration, stabilization, etc (Chaney et al., 2000). As one of the categories of phytoremediation, phytoextraction is the use of unusual hyperaccumulator plants to accumulate high quantities of metals in plant biomass. It offers a low cost strategy to clean up contaminated soils and the plant ash may also have economic value (Baker et al., 1994; Chaney et al., 2000).

Among the hyperaccumulator plants, *Thlaspi caerulescens* is the most extensively studied. *Thlaspi caerulescens* is primarily a Zn and Cd hyperaccumulator. It is an endemic metallophyte (i.e. an ancient colonizing species that is only competitive on contaminated sites) (Brooks, 1998). It actually requires abnormal amounts of Zn to be able to grow normally (Shen et al., 1997). Concentrations can exceed 3% and 0.1% of Zn and Cd, respectively, in shoot dry matter. The accumulation rates vary among populations of *Thlaspi caerulescens* (Perner et al., 2002), and the physical and chemical characteristics of the soils. The hyperaccumulation process involves a rapid uptake rate, high rates of translocation from roots to shoots, and huge storage capacity by vacuolar compartmentalization (Chaney et al., 1997). The first step is the rate-limiting step, uptake is confined by metal availability. Increasing metal availability usually results in enhanced uptake and higher shoot metal concentration (Brown et al., 1995 a).

2.2.2 Metal bioavailability and *Thlaspi caerulescens* hyperaccmulation

Chemical fractionation procedures have been proposed as a means to identify plant available forms of heavy metals in soil. Different sequential extraction procedures (SEP) have been used to partition metals into fractions as soluble, exchangeable, adsorbed, organically bound, oxide-bound, precipitated, occluded and residual (Davidson et al., 1994; Welter et al., 1999). Researchers have for many years tried to correlate metals in these fractions with plant concentrations (Tsadilas et al., 1995; Sims and Kline, 1991). Although SEP is useful as an indicator of metal bioavailability, correlati on studies are of less value. Metal bioavailability only correlated with plant tissue concentration when it is a limiting factor for plant uptake due to lowsoil buffering capacity or low plant solubilization. But in most cases, especially for *T. caerulescens*, metals released from formerly non-available forms reached more than 50% of the metals accumulated in plants (Knight et al., 1997 a; Whiting et al., 2001 a; Whiting et al., 2001 b). Thus, the dynamic cyclic process: depletion due to plant uptake and replenishment due to solubilization and desorption are generally not equilibrated. Measured metal concentrations can only capture a "moment in time" while plant metal concentration is an accumulation of uptake over time. In mathematical terms, it is an integration of numerous "moments" of metal concentrations. This may explain why so many discrepancies exist with similar studies.

2.2.3 Previous investigations related to adjusting soil pH and *T. caerulescens* hyperaccumulation

Phytoavailability of metals is strongly controlled by soil factors, such as pH, cation exchange capacity, organic matter content, oxides content, etc. Theoretically, lowering

pH will increase metal availability. Studies conducted on other crops have shown a negative correlation between soil pH and metal transferred to plants (Narwal et al., 1983; Castilho and Chardon, 1995). Only a few studies have investigated the pH effect on *T. caerulescens* hyperaccumulation (Brown et al, 1994; Brown et al., 1995 b) (Table 1). The causal relationship between soil pH and *T. caerulescens* accumulation and whether adjusting soil pH is an efficient way to enhance *T. caerulescens* hyperaccumulation requires further study.

Table 2.1. Influence of pH on *T. caerulescens* hyperaccumulation

Study Type	pH levels	Zn mg kg^{-1}	Cd mg kg^{-1}	Toxic pH [1]	Toxic [metal][2]	Optimum pH[3]	Source
Green-House	3	48000 4100 2100	1020 38 38	5.81	Zn 4100 Cd 38	6.67 5.42 6.82	Brown et al., 1994
Field	2	181-48	5.5-0.3	5.2			Brown et al., 1995 b

Note: 1, toxic pH-the highest pH when plant had significant yield reduction at lowest metal concentrations.
2, toxic [metal]-the lowest metal concentrations when plant had significant yield reduction at highest pH.
3, optimum pH-the pH when plant had extracted highest amount of metals (biomass × metal conc.) within each treatment

In the greenhouse study, at a soil concentration of Zn 48000 mg kg^{-1}, lowering pH increased shoot Zn concentration, but since shoot yield was also reduced, the total Zn translocated to plant biomass was actually lower. Highest uptake occurred at the medium pH treatment. At the lowest Zn soil content, *T. caerulescens* yield was negatively influenced by lowering pH and hence the total Zn extracted. For Cd, low pH reduced

yield, thus total Cd translocated to *T. caerulescens* was highest at the highest pH treatments at the two highest metal concentrations. For the lowest metal soil, uptake was highest at the medium pH treatment (Brown et al., 1994). No consistent result was observed for either metal concentration in biomass or total metal extracted by plant. In the field study, soil pH had no effect on Zn uptake, but lowering pH increased Cd uptake at the two highest metal treatments. In the control and low metal treatments, there was no significant difference in uptake (Brown et al., 1995 b). A possible reason for the lack of difference is that lowering pH creating a trade-off between plant growth and metal uptake. Metal concentration in the greenhouse study was high and the negative effect on yield of lowering pH was dominant, while in the field study, the metal concentration was too low to observe a more apparent pH effect. Kayser et al. (2000) used a sulfur amendment and observed a more consistent effect of enhancing Zn and Cd uptake by other plant species, *B. juncea, N. tabacum, S. Viminalis, H. annuus, Z. mays. Thlaspi caerulescens* was too sensitive to survive in this experiment. The sulfur treatment caused a small decrease in soil pH, but a significant increase in Zn and Cd mobility. These authors therefore attributed the S effect to soluble salts rather than a direct pH influence.

2.2.4 Mechanisms by which *Thlaspi caerulescens* scavenging metals

Instead of avoiding metal polluted spots, *Thlaspi caerulescens* roots preferentially colonize Zn and Cd-polluted areas (Whiting et al., 2000). The allocation and morphology of roots are strongly influenced by Zn and Cd content and form in soil. When all roots were in homogeneous soil polluted with a soluble Zn salt ($ZnSO_4$), root growth was severely inhibited. The positive response of roots to metals is specific, only to Zn and Cd,

and there is no response to Pb (Schwartz et al., 1999). This specificity and precision of distribution of the root system is considered an important factor in determining the efficient removal of metals.

Once roots have proliferated in metal rich soil, there is still a problem that *T. caerulescens* has to overcome: how to make the metals available? Rhizosphere acidification and release of root exudates are two common mechanisms by which plants modify the rhizosphere to acquire nutrients. A study by Luo et al. (2000) investigated soil solution Zn and pH dynamics during phytoextraction of *T. caerulescens*. Soil solution pH decreased initially and then increased slightly in both planted and unplanted soil zones. From 60 to 84 days after transplanting, the pH of the rhizosphere soil solution was higher than that of non-rhizosphere soil solution. This indicated that rhizosphere acidification was not the primary mechanism for mobilization of Zn in soil for *T. caerulescens*. Similar result was found in a pot study (McGrath et al. 1997). In this study, the pH of the rhizosphere soil was 0.2-0.4 units lower than that in the non-rhizosphere soils. But compared with the non-hyperaccumulator *T. ochroleucum*, *T. caerulescens* did not acidify more of its rhizosphere. Root exudates do not appear to play a role in metal mobilization of *T. caerulescens* hyperaccumulation, either. (Zhao et al., 2001).

On the contrary, it was repeatedly found that *T. caerulescens* was able to get access to less soluble Zn fractions in soil. In McGrath's study, decreases in the mobile fraction of Zn accounted for less than 10% of the total uptake of *T. caerulescens*, that is, more than 90% of the Zn must have come from the non-mobile fractions (McGrath et al., 1997). These authors also found that rhizosphere soils tended to have higher concentrations of mobile Zn than the non-rhizosphere soils. Similarly, in a study by Knight et al. (1997),

the decrease of Zn in soil solution after growth accounted for only 1% of the total Zn uptake by *T. caerulescens.* The authors suggests that either *T. caerulescens* was highly efficient at mobilizing Zn which was not initially soluble, or the soil could replenish solution Zn rapidly due to high buffering capacity (Knight et al., 1997 a).

To test which one of the above two possible mechanisms is more important, Whiting et al. (2001 b) used cө cultivated plants to see if mobilization of Zn by *T. caerulescens* increases Zn concentrations of a co-cultivated indicator plants (*Thlaspi arvense* or *Festuca rubra*) provided that they shared the same rhizosphere. *Thlaspi caerulescens* did not increase Zn concentrations in either of the indicator plants, suggesting that *T. caerulescens* does not "strongly" mobilize Zn in its rhizosphere (Whiting et al., 2001 b). In another experiment, whiting et al. (2001 a) used five Zn compounds of different solubility (ZnS, $Zn_3(PO_4)_2$, ZnO, $ZnCO_3$, and $ZnSO_4 \bullet 7H_2O$) to test how Zn hyperaccumulation was influenced by Zn bioavailablity. In a Clough Wood soil, the use of Zn-sulphate resulted in greatest total Zn in plant biomass, while in a Prayon soil, highest uptake was from the Zn-oxide fraction. In the unenriched and ZnS enriched treatments, about 70% and 50% of *T. caerulescens* biomass Zn came from previously non-labile forms. But Zn hyperaccumulation in these two treatments was less than that from the other four treatments. Again, they argued that this indicated that the solubilization effect of Zn by *T. caerulescens* was not strong. But comparing the nitrate-extractable Zn in day 0 and day 90, there was a significant increase in all of the five Zn-enriched treatments. In the Zn-sulphide treatment, there was an almost 10-fold increase in both soils. If this was caused by *T. caerulescens*, it was obviously a very strong

solubilization effect. Unfortunately they did not study an un-planted treatment to exclude the possible effect due to incubation.

Hutchinson et al. (2000) used another approach to test if *T. caerulescens* can use a non-labile pool of soil Cd by comparing [CdL] and [CdE] values using isotopic dilution techniques. [CdL] is the labile or bioavailable Cd determined from the specific activity of ^{109}Cd and the concentration of Cd in the plant leaves{ [CdL] = ^{109}Cdsoil (Cdshoot/^{109}Cdshoot)}, while [CdE] is the concentration of labile soil Cd determined chemically using a ^{109}Cd distribution coefficient. Comparing [CdL] and [CdE], [CdL] > [CdE] may indicate mobilization of non-labile Cd or that the isotope had gradually mixed with the non-labile pool of metal during the experiment. [CdL] < [CdE] may indicate non-thorough mixing of ^{109}Cd within the labile pool of soil Cd. For most of their treatments, the ratio of [CdL]:[CdE] were close to 1 indicating that *T. caerulescens* did not mobilize non-labile forms of Cd in soil. This conclusion was invalid unless they assumed that there was negligible fixation of ^{109}Cd, the argument was made by the authors based on the close agreement between ^{109}Cd and 1M $CaCl_2$ extractable Cd. But there was no evidence that added radiolabile Cd could all be extracted by $CaCl_2$. Ahnstrom and Parker (2001) found that even in sorbed /carbonate fractions, 70-75% of Cd was isotopically labile, while within oxidizable fraction, 35-41% of the Cd was labile. The contribution of $Sr(NO_3)_2$ extractable Cd (which was comparable to $CaCl_2$ extractable in the Hutchinson et al's study) to the radiolabile ^{109}Cd was only 1, 5, 14% in three of the soils, respectively. Only in one soil did it ever reach 75%. Therefore the [CdL] value was overestimated in this experiment, the true value should be smaller than [CdE], indicating non-thorough mixing of ^{109}Cd within the labile pool of soil Cd.

2.3 SOIL MICROBIAL ECOSYSTEM

2.3.1 Major groups of soil microorganisms

Bacteria, actinomycetes, and fungi are three major groups of soil microbes. Among them, bacteria are considered the most abundant and active group. Numerous environmental factors govern the activity and composition of bacteria populations. The optimal growth requires a nearly neutral pH for most species. Acid conditions inhibit the growth of many common species. Based on their morphology, bacteria can be divided into three major types, the rod shaped, the spherical-shaped, and the spirals (Alexander, 2005). The formation of endospores by some of the bacilli provides a strategy to survive under adverse environment (Alexander, 1961).

Actinomycetes are perceived as a transitional group between simple bacteria and fungi. By definition, the actinomycetes are “unicellular microorganisms that produce a slender, branched mycelium which may undergo fragmentation or may subdivide to form asexual spores” (Alexander, 1961). They share some common characteristics with bacteria, such as the morphology and size; while some others with fungi, such as the slow growth rate, the branching nature of their mycelium, etc. Actinomycetes generally prefer neutral to slight alkaline environment and are not tolerant of acid conditions. pH 5 is believed to be the threshold for most strains to survive.

Although not as abundant as bacteria in the term of numbers, fungi may contribute most to the total soil microbial protoplasm due to large size and extensive network of filaments. The forming of filamentous mycelium network of hyphal strands is one of the most prominent characteristics of fungi. pH, among many other environmental factors, is one of the major ecological variables controlling the growth and activity of fungi (Morton,

2005). However, it has been reported that many fungi species can tolerate over a wide pH range (Alexander, 1961). Therefore soil fungi tend to dominant at low pH due to lack of competition from bacteria and actinomycetes. Responding to a number of environmental modifying factors, the composition of the total soil microbial community is dynamic and under constant changes.

Soil is also the habitat of a large number of indigenous viruses. The number may be as high as 10^{11} g^{-1} of soil. Viruses are very small – most virus particles are in the range of 30 to 200 nm. They are usually composed of two basic components: a protein coat and genetic material, either DNA or RNA. All living organisms are susceptible to viral infection. Based on the host they infect, viruses can be classified into bacteriophages, plant viruses, animal viruses, insect viruses, and fungal viruses (Farrah and Lukasik. 2005).

Other soil microbial components include archaea, cyanobacteria, algae and soil fauna (Alexander, 2005; Belnap, 2005; Amador and Görres, 2005).

2.3.2 Soil microbial-mediated processes

The most important function of soil micro-organisms is decomposition of organic matter, a process by which the biological carbon cycle starting from photosynthesis is completed and CO_2 is replenished. Plants contribute most to soil organic carbon. Plants contain 15-60% cellulose, 10-30% hemicellulose, 5-30% lignin, and 2-15% protein, and about 10% of soluble substances, such as sugars, organic acids, and amino acids (Paul and Clark, 1989). In addition, dead cells of microbes and biosolids and animal manure all provide carbon to soils (Wolf and Wagner, 2005). Carbon decomposition is a successive

process. In the first stage, easily mineralized components are assimilated into microbial biomass and about half of the carbon is released as CO_2 into the atmosphere. In the second stage, cellulose and carbonhydrates, as well as microbial biomass formed during the first stage are degraded and transformed into new microbial biomass and half of the carbon is released as CO_2. The third stage, more resistant substrates which are high in lignin and aromatic rings are utilized by microbes and release about two-thirds of the carbon as CO_2 (Wolf and Wagner, 2005). The residue components, mainly humus, are very resistant to decomposition.

Nitrogen is an essential nutrient for all life and is the most limiting nutrient for plant growth in terrestrial ecosystems (Myrold, 2005). It is the fourth most common element in plant biomass composition (Paul and Clark, 1989). Nitrogen is present in different forms: dinitrogen gas (N_2), organic nitrogen, ammonium (NH_4^+), and nitrate (NO_3^-) (Myrold, 2005). Shifts between different forms are carried out by soil microbes. Major nitrogen transformations in the nitrogen cycle include ammonification, immobilization, nitrification, dissimilatory NO_3^- reduction, denitrification, symbiotic N_2 fixation, nonsymbiotic N_2 fixation, plant uptake of NH_4^+ and NO_3^-, decomposition of plant and animal residues to organic N (Myrold, 2005; Zubber, 2005; Graham, 2005).

Sulfur (S) is an essential element for growth and activity for all living organisms and plays many important biological functions (Germida, 2005). Sulfur atoms are found in many organic and inorganic compounds and are important components of soil organic matter, microbial biomass, and soil minerals (Tate, 1994). It exists in a wide range of oxidation states in various compounds, such as organic S (R-SH), sulfide (S^{2-}), elemental S (S^0), thiosulfate ($S_2O_3^{2-}$), sulfur dioxide (SO_2), sulfite (SO_3^{2-}), and sulfate (SO_4^{2-}) (Paul

and Clark, 1989). Transformations of S-bearing compounds with different oxidation states serve as both energy sources and electron acceptors for soil microbes (Tate, 1994). Microbial oxidation of elemental S into sulfate and microbial oxidation of inorganic S compounds both have important environmental consequences (Germida, 2005).

Phosphorus is a critical component of many important biomolecules such as DNA (deoxyribonucleic acid), phospholipids, and ATP (adenosine triphosphate) (Mullen, 2005). Phosphorus exists primarily in either insoluble or only very poorly soluble inorganic forms, mainly rock phosphate, or apatite (Paul and Clark, 1989). Phosphorus can be affected by both biological and chemical reactions. Chemical weathering of apatite releases orthophosphate. In the biological phosphorus cycle, orthophosphate can be taken up by plants or immobilized into microbial biomass. Biomass phosphorus can then be incorporated into soil organic matter and subject to many other mineralization and immobilization reactions (Mullen, 2005).

Soil microbes are important in transformation of many other elements as well, including iron (Fe), manganese (Mn), arsenic (As), mercury (Hg) and selenium (Se) (Mullen, 2005).

2.3.3 Environmental factors that control and influence soil microbial activities

Numerous environmental factors affect the growth and activity of soil microbes. Soil microbes, as soil-inhabiting microorganisms, are affected by soil physical components such as texture, mineral composition, organic matter content, and soil aggregation. Soil microbes can be adsorbed on clay surfaces or by humic substances thereby providing protection and modification of activities (Yates and Yates, 1988). Heterogeneity of the

soil environment contributes to a diverse soil microbial population and provides niches for microorganisms to survive under adverse environmental conditions.

The growth and reproduction of soil microorganisms require energy, electron acceptors, macronutrients, and micronutrients. The abundance, availability and distribution of these elements govern soil microbial population dynamics. Soil heterotrophs use C-compounds formed by photosynthesis as the primary energy source. Under normal conditions, O_2 is the common electron acceptor. Under anaerobic conditions, microbes can use other alternatives such as NO_3^-, NO_2^-, or SO_4^{2-}. The most important macronutrients for soil microbes are C, N, P, and S. Microorganisms also require Fe, Mg, Mo, Zn, etc. as enzyme co-factors or to fulfill other metabolism functions (Alexander, 2005).

Soil water content affects the abundance of soil microorganisms (Stotsky, 1997). Water is needed in a number of cellular metabolic processes and is an essential medium for growth. Soil moisture also greatly affects nutrient availability and transport. Soil aerobic and anaerobic conditions are determined by the relative amount of water and air in the soil pores.

Soil pH has great impact on soil microbial growth and activity. Most soil microbes have an optimum pH and only grow and function within a certain pH range. Extreme pH will adversely affect both population development and activity (Crane and Moore, 1986). In addition, pH is a key factor determining nutrient availability and soil heavy metal toxicity. It has been reported that as long as pH is maintained above 7, there are few observed adverse effects of elevated heavy metal concentrations. However, at low pH,

the lowest metal concentration where an adverse effect was observed was much lower. Low pH will also induce Al toxicity and Mo deficiency at some soils (Sparks, 1995)

According to the temperature that microorganisms can grow, they are divided into thermophiles-which only grow at high temperature, typically 45-75^{o}C, psychrophiles-which grow only with a temperature below 20^{o}C, and mesophiles-which grow within the temperature of 15-45^{o}C and with an optimum temperature of around 30^{o}C. For most microorganisms, high temperature will cause protein denaturation, enzyme inactivation, increased membrane permeability and even cell wall rupture (Stotzky, 1997). Low temperature will reduce cell metabolism, activity, and growth rate.

Chapter 3 pH Effects on Distribution and Plant Uptake of Zn and Cd

ABSTRACT

For phytoextraction to be successful and viable in environmental remediation, the strategies that can optimize plant uptake must be identified. Whether adjusting soil pH is an efficient way to enhance *T. caerulescens* hyperaccumulation must by clarified. This study used two soils differing in levels of Cd and Zn and was adjusted to 5 or 6 different pH levels. Metals were extracted into 5 sequential fractions and the pH effect on the mobilization of metals from each fraction and *T. caerulescens* phytoextraction was assessed. Reducing pH redistributed Cd and Zn among the five fractions. The most soluble metal form (F1) was greatly increased with decreasing pH. Sequentiallymore recalcitrant fractions F2, F3, F4, and F5 all had different degrees of mobilization at low pH. Most of the "new" mobile Cd was from F2 and for Zn it was mainly from F2 and F3. Reducing pH significantly influenced plant growth and metal uptake. For the high metal soil, plants grew best at the lowest soil pH (4.74). The highest metal concentration was at the second lowest pH (5.27). For low metal soil, due to low pH induced Al and Mn toxicity, both plant growth and metal uptake was greatest at intermediate pH level. Plant uptake of metal also modified the rhizosphere soil metal environment. *Thlaspi caerulescens* was able to reduce Cd concentration in all 5 fractions, although F1-F3 were most significantly reduced. For Zn, *T. caerulescens* significantly reduced metal concentration in F1 and F2 pools, while no significant changes in F3-F5 pools were observed. Overall, reducing pH is an effective strategy to enhance phytoextration. However soil pH is not "the lower the better", a different optimum pH may exitst for each individual soil. This pH should be identified to avoid unnessarily extreme acidification treatment.

Key words: *Thlaspi caerulescens*, Cd, Zn, pH, Sequential extraction, Rhizosphere

3.1 INTRODUCTION

Phytoextraction uses unusual hyperaccumulator plants to remove contaminants from soil (Chaney, 1983; Baker and Brooks, 1989). As a promising alternative soil remediation technology, it has been the focus of extensive research over the past decade. Although the mechanisms of hyperaccumulation remains unclear, it is generally agreed that hyperaccumulation involves three major processes: rapid uptake of heavy metals by roots, high rate of translocation from roots to shoots, and high storage capacity by vacuolar compartmentalization (Chaney et al, 1997).

Among known hyperaccumulator plants, *Thlaspi caerulescens* is the most extensively studied. It is an endemic metallophyte (i.e. an ancient colonizing species that is only competitive on contaminated sites) and primarily a Zn hyperaccumulator (Brooks, 1998). *T. caerulescens* actually requires abnormal amounts of Zn to be able to grow normally (Shen et al., 1997). Concentrations can exceed 3% and 0.1% Zn and Cd, respectively, in shoot dry matter. Accumulation rates vary with plant genotypes (Perner et al., 2002), and physicochemical characteristics of soil.

Mechanisms by which *T. caerulescens* scavenges metals from soils are not fully understood. Studies have suggested that specific and precise distribution of roots is an important factor in determining the efficient removal of metals by *T. caerulescens* (Whiting et al., 2000; Schwartz et al., 1999). Once roots have proliferated in metal rich soil, the concentration of soluble and plant available metal must be high enough to meet the extraordinary requirement of *T. caerulescens.* Rhizosphere acidification and releasing

of root exudates are two common mechanisms by which plants modify the rhizosphere to acquire nutrients. However, Luo et al. (2000) found that rhizosphere soil had higher pH than non-rhizosphere soils. This suggests that rhizosphere acidification is not an important mechanism to mobilize Zn in soil for *T. caerulescens*. Similar results were found in a pot study by McGrath et al. (1997). *Thlaspi caerulescens* was able to access Zn from less soluble fractions in soil although it does not "strongly" mobilize Zn in its rhizosphere (Whiting et al., 2001 b). If *T. caerulescens* is not able to mobilize non-labile metals, then uptake will depend on the soils potential to replenish metal supply.

There are numerous chemical processes in soils that influence metal solubility. pH is the most important factor. For example, heavy metals can be retained by the permanent charge sites of layered silicate clays through non-specific electrostatic forces or specific chemisorption. The irreversibility and the specificity are increased at higher pH (Farran and Pickering, 1976, 1977; Tiller et al., 1979, 1984). Two-dimensional surface adsorption of metals by oxides, hydroxides, and amorphous alluminosillicates is partially or completely reversible by pH change. Lowering pH, therefore, usually results in greater uptake by plants.

Sequential extraction procedures use a series of chemical reagents with increasing strength to extract metals. They provide a useful and important technique to understand the geochemical distribution, mobility, and biological availability of metals. Since total soil metal concentration has little association with phytotoxicity, it is important to quantify the various fractions of metals in contaminated soils.

Studies conducted on other crops have shown a negative correlation between soil pH and metal uptake (Narwal et al., 1983; Castilho and Chardon, 1995). Studies on pH

effect on metal uptake by *T. caerulescens* hyperaccumulation, however, are lacking (Brown et al, 1994; Brown et al., 1995 b). For phytoextraction to be successful and viable in environmental remediation, the strategies that can optimize plant uptake must be identified. Whether adjusting soil pH is an efficient way to enhance *T. caerulescens* hyperaccumulation must by clarified. Therefore the primary objective of this work was to examine the effects of pH on metal availability and *T. caerulescens* hyperaccumulation.

3.2 MATERIALS AND METHODS

3.2.1 Site description and soil sampling

Soil samples were collected from the A horizon of two cultivated fields near a former Zn smelter that had been in operation for nearly 100 years at Palmerton, PA. Metals released to the environment were primarily Zn and Cd resulting in a metal concentration gradient according to the distance and direction from the smelter. Two soils were sampled, one was about 4.5 km up wind from the smelter and was characterized by relatively low metal concentrations; The other soil was collected about 1.4 km down wind from the smelter, and contained higher metal content (Table 3.2). Both soils belong to Montevallo series (loamy-skeletal, mixed, subactive, thermic, shallow Typic Dystrudepts). Soils were first passed through a 1 cm sieve to remove stones and large plant residues then passed a 4 mm sieve. Soils were then homogenized and stored in closed containers to avoid dehydration.

3.2.2 Soil characterization

Total Zn and Cd concentrations were measured by extracting with concentrated hot nitric acid and measured by flame atomic absorption spectrometry. Soil particle size distribution was determined by the hydrometer method (University of Maryland, 1978.). Soil pH was measured in a soil water suspension (10 g soil to 20 ml deionized water) after shaking 1 h at 180 rpm on a reciprocal shaker. Organic matter content was determined by loss on ignition. Plant available Ca^{2+}, Mg^{2+}, and K^{+} were extracted with Mehlich (I) [M (I)] and determined on a Technicon Auto-Analyzer using a colorimeter for Mg and a flame photometer for K and Ca. Total N was determined by the combustion method. Plant available P was extracted with Mehlich (I) and determined using a Technicon Auto-Analyzer.

3.2.3 Soil pH adjustment and salt leaching

Different amounts of elemental sulfur (S) were used to adjust soil pH to desired levels based on a preliminary acid incubation experiment. Soil pH was monitored periodically by taking 10 g soil sample and measuring pH. Soil was thoroughly mixed every day to ensure equal distribution of S and to accelarate the S oxidation process. Incubation was terminated when pH did not change for 3 consecutive weeks. Next, 500 ml of deionized water was added to each pot to leach salts from soil. This procedure was repeated two additional times.

3.2.4 Plant growth

Thlaspi caerulescens used in this research is a southern France type, collected from Viviez, France with very high Cd hyperaccumulation potential (Chaney, personal communication). Seeds were germinated and seedlings were grown for 60 days and watered daily to maintain relatively constant moisture. The flats were put into a controlled-environment growth chamber, which was set at 16h/8h day/night cycle at $25^{o}C/22^{o}C$. Light intensity was above 400 µmol photon m^{-2} s^{-1} and relative humidity was 65%. Peters™ 20-20-20 general purpose fertilizer was used as liquid spray when needed. Seedlings were then transplanted into 15 cm (diameter) by 14 cm (height) plastic pots. Each pot contained 1 kg soil and received three plants. Pots were put into growth chambers with the same settings as for the seedlings growth. After transplanting, the use of fertilizers was limited to avoid disturbing soil microbial systems. After another 6 months of growth, plants were harvested.

3.2.5 Rhizosphere soil sampling

Rhizosphere soil is defined as that portion of soil adjacent to and influenced by plant roots (Metting, 1993). *Thlaspi caerulescens* has a very prolific root system. After 6 months of growth, all the soil in the pot was filled with fine roots and considered as rhizosphere soil. At harvest, the shoots were cut at the base using stainless steel scissors. The whole soil/root mass was removed from the pot. Root and soil were manually separated.

3.2.6 Experimental design

A completely randomized block design with treatment in factorial combination was used with the following factors: 1) metal concentration (low and high), 2) presence of plant (w/ and w/o plant, i.e., rhizosphere soil and non-rhizosphere), and 3) soil pH (6.88, 6.37, 6.07, 5.28, 4.74). For the low metal soil, soil pH was adjusted to 6 levels; an additional pH treatment of 7.27 was used. There were 4 replications for each of the treatments which were randomly put into one of the four growth chambers.

3.2.7 Sequential extraction procedure

Prior to extraction, 6-8 g of each soil was air-dried overnight, and ground to pass a 150 µm sieve. Duplicate 2 g samples were added to 50 ml polycarbornate centrifuge tubes and sequentially extracted into five operationally defined fractions (Table 3.1).

Between each fraction, a 5 ml 0.1 M NaCl rinse was used and pooled with the preceding extract. Concentrations of Cd and Zn in the F2-F5 fractions were determined using a flame atomic absorption spectrometer. The detection limits (DL) were 0.015 µg g^{-1} and 0.050 µg g^{-1} for soil Cd and Zn, respectively. Concentrations of Cd and Zn in F1 were determined using an inductively coupled plasma spectrometer. Laboratory standards were routinely included in analysis.

3.2.8 Plant biomass metal extraction

Plant shoot and root tissue were separately washed in deionized water, and dried at 70^{o}C. Shoot tissue was grounded when it weighed more than 4 g. Dry plant biomass was weighed and ashed in a muffle oven at 480^{o}C for about 16 h. After cooling, 2 ml concentrated HNO_3 was added to the beaker. Beakers were then placed on the surface of

a hot plate and allowed to evaporate for 1 h to near dryness. Then 10 ml of 3 N HCl was added and the beaker was covered with a watch glass and refluxed on a hot plate for 2 h. The mixture in the beaker was then filtered into a 25 ml volumetric flask through a Whatman #40 filter paper. 0.1 N HCl was added to volume. Yttrium was added as an internal standard. Element concentrations were determined using an inductively coupled plasma spectrometer. National Institute of Standards and Technology (NIST) plant standards were included in analyses.

3.2.9 Statistical analysis

Statistical analyses were conducted using SAS version 8.2 (SAS Institute, 2001). The assumption of normality was tested by examining the plot of residuals and calculating the Shapiro-Wilk statistic. The homogeneity of variance was assessed by examining a plot of predicted values versus residual values. The Spearman test was used to test the significance of the correlation between the predicted value and absolute value of the residual. Logarithm transformation of data was performed for some variables when needed. After checking that data met the assumptions, the PROC MIXED procedure was used for univariate ANOVA to determine the significance of the main factors and their interactions with block as a random factor, the pH treatment of 7.27 in the low meal soil was omitted when doing this analysis. When significant effects were detected, pair-wise treatment mean comparisons were made using a Least Significance Difference (LSD) t-test on pH treatment means. Linear or quadratic regression equations were calculated by the least-squares method. Differences between non-rhizosphere soil and rhizosphere soil treatment means were compared by a paired t-test. The association between two variables

was estimated by the Pearson product-moment correlation coefficient. Unless otherwise indicated, all the statistical significance levels were set as $p \leq 0.05$.

3.3 RESULTS

3.3.1 Plant yield

Metal and pH by metal interaction had a significant effect on yield of *T. caerulescens* (Table 3.3). For the high metal soil, plant dry weights ranged from 5.1 to 6.8 g and highest shoot yield was at the lowest pH treatment. This may be related to increased metal concentrations at the lower pH treatment. As previously noted, abnormally high concentrations of metal (Zn) are required by *T. caerulescens* in order to grow well.

For the low metal soil, highest yield was observed at pH 6.07. The lowest pH treatment showed a dramatic yield reduction. Plant growth at the lowest pH treatment was also noticeably slower with a much smaller rosette, and fewer leaves. The root development of the plant in the lowest pH treatment of the low metal soil was also characterized by an unhealthy, stunted root system, lacking small side branches and fine roots. This is a typical symptom of Al toxicity. Metal extraction showed that 0.1 M $Sr(NO_3)_2$ extractable Al was 8 to 10 fold higher in the lowest pH treatment in the low metal soil.

3.3.2 Effect of pH on biomass Cd concentration

Plants grown in the higher metal soil had much higher shoot Cd concentration than those in the lower metal soil. pH also had a significant effect on shoot Cd concentration.

For the higher metal soil, shoot Cd concentration ranged from 937-1456 mg kg^{-1} dry weight. The highest concentration was observed at pH 5.28. There was no significant difference between the three higher pH treatments (Fig 3.2 a). For low metal soil, shoot Cd concentration ranged from 86-355 mg kg^{-1} dry weight. The concentration was highest at pH 6.07 and lowest at pH 4.74. Cd concentration increased with decreasing pH from pH 7.27-6.07, then rapidly decreased in the lower pH treatment (Fig 3.2 b).

Unlike the shoot, root Cd concentrations did not respond to pH change. For all pH treatments, the root Cd concentrations were not significantly different from each other. However, the high metal soil still had much higher root Cd concentrations than the low metal soil. Although not statistically significant, root Cd tended to increase with reduced soil pH in the high metal soil. The highest concentration of root Cd was observed at the lowest pH with a value as high as 1472 mg kg^{-1} dry wt. Surprisingly, the root concentrations were similar to shoot concentrations. For high metal soil, concentrations ranged from 802-1472 mg kg^{-1} while for low metal soil, they ranged from 136-272 mg kg^{-1}. This was contrary to the current belief that for hyperaccumulators, the shoot concentration usually will be much higher than the root concentration. The root Cd concentration was even higher than the shoot in the lowest pH treatment for both of the soils implying that the extreme low pH has hampered the root ability to translocate Cd from root to shoot.

3.3.3 Effect of pH on biomass Zn concentration

pH had a significant effect on the shoot Zn concentration. For high metal soil, shoot Zn concentrations ranged from 3986-5259 mg kg^{-1} dry weight. The highest concentration

was at pH 5.28, the lowest concentration was at pH 6.07 (Fig. 3.3 a). For low metal soil, shoot Zn concentration ranged from 1314-5642 mg kg^{-1} dry weight. The highest concentration was at pH 6.07, apart from this pH value, shoot Zn decreased with increasing distance from the optimal pH. The lowest concentration was observed at pH 4.74 (Fig. 3.3 b).

Similar to root Cd, root Zn concentration was not significantly affected by pH. For high metal soil, root Zn concentration ranged from 744-1611 mg kg^{-1} dry weight. The lowest concentration was at pH 6.07, which was the same as shoot Zn. However, the highest concentration was at pH 4.74, again, implying the impeded translocation ability at the lowest pH treatment. Except the lowest pH treatment, there was no significant difference between the other four pH treatments. For low metal soil, root Zn concentration ranged from 376-977 mg kg^{-1} dry weight. The highest concentration was at pH 6.07, which was also seen for the shoot Zn. But the lowest concentration was at pH 7.27.

3.3.4 Effect of pH on total Cd accumulated in shoot

Reducing pH significantly increased the total Cd accumulated in shoots. For the high metal soil, the value ranged from 5.8 to 9.1 mg pot^{-1}. The highest extraction was at pH 5.28. The second highest one was at pH 4.74. Both were significantly higher than the other three higher pH treatments (Fig. 3.4 a). For the low metal soil, the values ranged from 0.2 to 2.0 mg pot^{-1}. The highest extraction was at pH 6.07. Total Cd extraction at pH 5.28, 6.37, 6.88, and 7.27 were not significantly different. However, when pH was reduced to 4.74, there was a drastic reduction in total Cd phytoextraction (Fig. 3.4 b).

This was due to the combination of significant yield reduction and lowered metal concentration in the shoot. There was also a significant difference between the two tested soils. Plants grown in the high metal soil extracted much higher Cd than those in the low metal soil at all pH treatments. Amazingly, *Thlaspi caerulescens* extracted up to 9.08 mg Cd in the high metal soil pH treatment of 5.28. Considering the total soil in the pot weighted 1 kg with total Cd concentration of 24 mg kg^{-1}, a single harvest of *T. caerulescens* was capable to phytoextract about 38% of the total soil Cd indicating great potential for Cd remediation.

3.3.5 Effect of pH on total Zn accumulated in shoot

Total Zn phytoextracted to shoots followed a similar pattern as Cd. For the high metal soil, the values ranged from 17 to 27 mg pot^{-1}, which was about 1-2% of total soil Zn. The highest extraction was at pH 5.28, the second highest at pH 4.74 (Fig. 3.5 a). For low metal soil, the value ranged from 12 to 32 mg pot^{-1}, about 5-8% of the total soil Zn. Highest extraction was at pH 6.07; lowest extraction was at pH 4.74. There were no significant difference between pH treatments of 5.28, 6.37 and 6.88. However, the control treatment was significantly lower than the other pH treatments except for pH 4.74 (Fig. 3.5 b).

3.3.6 Effect of reducing pH on *T. caerulescens* uptake of other nutrients and heavy metals

pH had a significant effect on shoot Ca concentration (Table 3.4). For high metal soil, the highest concentration was at pH 5.28. There was no significant difference

between the other four pH treatments (Fig. 3.6 a). For low metal soil, the highest concentration was at the second highest pH level, 6.88, after that, Ca concentration decreased with descending of pH (Fig. 3.6 b). Root Ca, however, was not affected by pH in general. For high metal soil, no significant difference was observed between all the five pH treatments. For low metal soil, similar to shoot Ca, the highest value was at pH 6.88 while the lowest value was at pH 4.74. Shoots generally had 1-3 times as much Ca as roots.

Thlaspi caerulescens had very limited ability to accumulate Cu from soil. The shoot concentration of Cu was very low. For high metal soil, it ranged from 2.1-2.9 mg kg^{-1} dry weight, for low metal soil, it ranged from 2.2-4.5 mg kg^{-1} dry weight. Overall, pH and soil type had significant effect on uptake. Shoot Cu concentration tended to increase with decreasing pH. For high metal soil, the highest value was at the lowest pH treatment, however, there was no significant difference between all five pH treatments (Fig. 3.7 a). For low metal soil, the highest concentration occurred at pH 5.28, and the second highest was at pH 4.74. There was no significant difference between the other four higher pH treatments (Fig. 3.7 b). It is interesting to note that, root Cu concentration was much higher than shoot. It ranged from 8.2-12.5 mg kg^{-1} dry weight. pH did not have significant effect on root Cu concentration. The high metal soil had higher root Cu concentrations than the low metal soil.

Shoot Fe concentration was also significantly affected by pH and soil type. Concentrations tended to increase with decreasing pH. For this metal, the low metal soil generally had higher concentrations than the high metal soil, especially in the lower pH treatments. There were no significant differences between all five pH treatments in the

high metal soil (Fig. 3.8 a). For low metal soil, the three higher pH treatments had lower concentrations than the three lower pH treatments. And the highest value was at pH 4.74 (Fig. 3.8 b). Overall root Fe concentration was not affected by pH. There were no significant differences between all five pH treatments in the high metal soil. However, in the low metal soil, root Fe concentration presented an irregular pattern, being highest at pH 7.27, lowest in the middle pH, and then increased again at the lowest pH. Similar to Cu, root Fe concentration was much higher than the shoot. The former ranged 615-1799 mg kg^{-1} dry weight, while the latter ranged 47-123 mg kg^{-1} dry weight.

Shoot Mn concentration was significantly affected by pH, soil type and their interaction. For the high metal soil, shoot Mn increased with decreasing pH, especially at the lowest pH where the concentration increased sharply (Fig. 3.9 a). From the highest pH to lowest pH there was a more than 10 fold increase in shoot Mn concentration. For the low metal soil, a sharp increase of Mn concentration occurred at the two lowest pH treatments. And from the highest pH to lowest pH there was a nearly 20-fold increase in shoot Mn concentration (Fig. 3.9 b). Plant grown in the low metal soil had higher Mn concentrations than those in the high metal soil. Root Mn concentration was also significantly affected by pH in a similar pattern as shoots, although the degree of increase in the lowest pH was not as great. At high pH levels, shoot Mn concentration were similar or even smaller than root concentration, however, at the lowest pH level, shoot Mn concentration was much larger than the root concentration.

Shoot Mg was significantly affected by pH, soil type and their interaction. For high metal soil, the highest value was at pH 6.37, then decreased with descending pH (Fig. 3.10 a). For the low metal soil, Mg concentration increased with the decrease of pH,

reaching a peak at pH 5.28, then decreased at the lower pHs (Fig. 3.10 b). pH also significantly influenced root Mg concentration. For both soils, the highest value was at the highest pH, while the lowest value was at the lowest pH. For this metal, shoot concentration was close to root concentration with slight variations with pH change.

Shoot K concentration was significantly affected by pH. For both soils, K levels in the shoot tended to increase with decreasing pH. The highest concentration was observed at the two lowest pH values (Fig. 3.11 a, b). However, root K did not respond to pH changes. For both soils, there was no significant difference between all pH treatments. In addition, at each pH level, there was no significant difference between the two soil types. Shoot K concentration was always a little higher than root. In the two lowest pH treatments of the low metal soil, the shoot concentrations were the highest while root concentrations were the lowest among all the treatment implying possible enhanced translocation in the low pH.

3.3.7 Effect of reducing pH on biomass shoot/root element concentration ratio

It has been suggested that the shoot/root ratios of metal concentrations greater than 1 is an important characteristic of hyperaccumulators (Baker, 1981; Rascio, 1977; Reeves and Baker, 1984; Brown et al., 1995 a). It can also reflect the metal translocation from root to shoot capability of *T. caerulescens*. At different soil pH values, the shoot/root ratios have different orders. For high metal soil, the orders are ranked as follow:

At pH 6.88, the ratio order is Zn>Ca>K>Cd>Mg>Mn>P>Cu>Fe.

At pH 6.07, the ratio order is Zn>Ca>K >Mg>Cd >Mn>P>Cu>Fe.

At pH 4.74, the ratio order is Zn>Ca>>Mn>K >Mg>Cd>P>Cu>Fe.

Zn and Ca shoot/root ratios are about 1 at all pH levels, while P, Cu, and Fe shoot/root ratios are below 1 at all pH levels. Potassium, Mg, and Cd shoot/root ratios varied in the proximity of 1 (Figure 3. 13). For all of the elements, ratios were relatively constant at the first three pH treatments. From pH 6.07-4.74, elements for which the ratios decreased included Zn, Ca, Cd, and P. Zinc decreased markedly while the other three elements ratios only decreased moderately. At same pH range, elements whose ratios increased included Mn and Fe. However, K, Mg, and Cu ratios were relatively constant at this pH range. Changes in the values of shoot/root concentration ratio could suggest a shift in the hyperaccumulating mechanism. The ratio's decrease may suggest retarded translocation from root to shoot, implying some internal system damage at low pH, as seen for both Zn and Cd-the two elements that *T. caerulescens* can hyperaccumulate.

For low metal soil, the ratio orders are ranked as follow:

At pH 7.27, the ratio order is Zn>Ca>Cd >K >Mn >Mg >P>Cu>Fe.

At pH 6.07, the ratio order is Zn>Mn>Ca>Cd >K >Mg >P>Cu>Fe.

At pH 4.74, the ratio order is Mn>K>Zn>Ca >Mg>P>Cd >Cu>Fe.

Zn and Ca shoot/root ratios are about 1 at all pH levels, while P, Cu, and Fe shoot/root ratios are below 1 at all pH levels. K, Mg, and Cd shoot/root ratios varied in the proximity of 1 in relationship to pH changes (Fig. 3.14). For all of the elements, ratios were relatively constant at the first three pH treatments. From pH 6.07-4.74, elements for which the ratios decreased included Zn, Ca, Cd. At same pH range, elements for which the ratios increased were Mn, K, Mg, P, and Cu.

3.3.8 Correlations between shoot elements concentration

The correlation coefficients between shoot Zn and shoot Cd with pH were small and insignificant (Table 3.7). Shoot Ca had a positive correlation with pH, while shoot Mn, Fe, Cu, and Mg all had negative correlation with pH. Shoot Mn was the metal that most correlated with pH, $r = -0.70$, $p<0.0001$. Shoot Zn is most correlated with shoot Cd, $r = 0.61$, $p<.0001$. While shoot Cd is most correlated with shoot Mg with $r = 0.79$, $p<.0001$. Shoot Mn is highly correlated with Cu and Fe, with the former $r = 0.74$, $p<.0001$ and the latter $r = 0.58$, $p<.0001$. Except shoot Mn, shoot Fe also highly correlated with shoot Cu, with $r = 0.54$, $p<.0001$.

3.3.9 Effect of reducing pH on the concentrations of 0.1M $Sr(NO_3)_2$ extractable Al, Ca, Mg, and Mn from soils.

Our data demonstrated the concentration of 0.1M $Sr(NO_3)_2$ extractable Al, Ca, Mg, and Mn was strongly affected by soil pH. Changing pH significantly changed extactable concentrations. Decreasing pH drastically increased the concentration of Al and Mn while decreased the concentration of Ca and Mg (Figure 3.15-18). The Al concentration responding to pH treatments was not the same for the high and low metal soils. For high metal soil, from the highest pH to the lowest, Al increased about 30%. However, for low metal soil, there was 8 to 11 fold increase. The final concentration in the low metal soil reached 49 and 71.8 mg kg^{-1} for rhizosphere soil and non-rhizosphere soil, respectively. It appears that the high and low metal soils may have different mineralogy. This phenomenon is consistent with the higher buffering capacity of low metal soil previously observed in the S addition experiment. However, there was little difference in the

concentrations of Ca, Mn and Mg between these two soils. Rhizosphere soil generally had lower 0.1M $Sr(NO_3)_2$ extractable metal concentrations, indicating the uptake by the plant root lowered the available metal concentrations around the roots.

3.3.10 Effect of reducing pH on Cd bioavailability and distribution

0.1 M $Sr(NO_3)_2$ extractable Cd concentration (F1) was greatly increased with soil acidification (Fig 3.19 a, b). In the high metal non-rhizosphere soil, Cd concentration increased from 0.7 to 5.8 mg kg^{-1} from the original pH to the lowest pH treatment. Each lower pH treatment had significantly higher F1 Cd than the following higher pH treatment. F1 Cd concentration increased from below the detection limit to 1.2 mg kg^{-1} in high metal rhizosphere soil. Similarly, pH 6.07 brought F1 Cd concentraton from below the detection limit to 0.5 mg kg^{-1} for the low metal soil. There was a significant increase in concentration with each lower pH treatment in the low metal non-rhizosphere soil.

Reducing pH significantly reduced sodium acetate extractable Cd (F2) in both soils. Specifically, for high metal non-rhizosphere soil, the three highest pH treatments had significantly higher Cd concentrations than the pH 5.28 treatment, and which in turn, had significantly higher Cd concentration than the lowest pH treatment. For high metal rhizosphere soil, the three highest pH treatments had significantly higher Cd concentrations than the two lower pH treatments (Fig. 3.20 a). For low metal soil, there was no significant difference between the three highest pH treatments. However, from pH 6.37 to the lower pH treatment, each lower pH treatment has significantly reduced Cd concentration. For both low and high metal soils, Cd concentration was significantly higher in the non-rhizosphere soil than in the rhizosphere soil (Fig. 3.20 b).

The third fraction of Cd, 5% NaOCl extractable (F3), significantly decreased with reduced pH. For high metal non-rhizosphere soil, there was no significant difference between the four highest pH treatments. However, Cd concentration was significantly lowered at the lowest pH treatment. For high metal rhizosphere soil, Cd in the three higher pH treatments were significantly higher than the two lowest pH treatments (Fig. 3.21 a). For the low metal soil, there was no significant difference between the three highest pH treatments. However, beginning at pH 6.37, every lower pH treatment significantly reduced the Cd concentration compared to its previous treatment. For the low metal rhizosphere soil, the three higher pH treatments had significantly higher Cd concentrations than the three lower pH treatments. From pH 6.07 to 4.74, Cd concentration kept relatively constant (Fig. 3. 21 b). In the high metal soil, non-rhizosphere soil had significantly higher Cd concentrations than the rhizosphere soil at each pH treatment. This was also true for the low metal soil, except at pH 6.88 and 4.74, where this difference was not statistically significant.

The fourth fraction (F4), 0.4 M oxalate plus 0.1 M ascorbate extractable Cd had much lower concentrations than the previous fraction. Because for the low metal soil, Cd concentrations were below the detection limit, only Cd of the high metal soil is discussed here (Fig. 3.22 a, b). Overall, Cd concentration of this fraction was not influenced by pH. There was no significant difference between all five pH treatments for non-rhizosphere soil; neither was the pH regression significant. For rhizosphere soil, however, the three higher pH treatments were significantly higher than the two lower pH treatments. There was significant quadratic pH regression response for Cd concentration with a R^2 value of

0.94. Non-rhizosphere soil had higher Cd concentrations than rhizosphere soil. This difference was significant at pH treatment of 4.74, 5.28, and 6.37.

For the same reason as above, the last fraction, residual form of Cd (F5), is discussed here only for the high metal soil (Fig. 3.23 a, b). Both non-rhizosphere soil and rhizosphere soil Cd concentration were decreased with reduced pH. This relationship can be expressed by linear pH regression models with R^2 values of 0.92 and 0.93, respectively. Again, non-rhizosphere soil had higher Cd concentrations than rhizosphere soil, but this difference only significant at pH treatments of 6.07 and 6.37.

3.3.11 Effect of reducing pH on Zn phytoavailability and distribution

0.1 M $Sr(NO_3)_2$ extractable Zn (F1) was greatly increased with soil acidification (Fig. 3.24 a, b). In the higher metal non-rhizosphere soil, Zn concentration increased from 5.7 to 158 mg kg^{-1} from original pH to the lowest pH treatment. There was a more than 30-fold increase in F1 Zn concentration from the highest to the lowest pH treatment in the high metal rhizosphere soil. Similarly, pH 6.07 brought F1 Zn concentraton from below detection limit to 6.4 mg kg^{-1} and then there was significant concentration increase with each lower pH treatment in the low metal non-rhizosphere soil. Except treatments where Zn concentrations were below the detection limit, non-rhizosphere soil had significantly higher Zn concentrations than rhizosphere soil.

With decreasing pH, F2 Zn declined in both high and low metal soils (Fig. 3.25 a, b). From the highest pH treatment to pH 6.07, this change was not statistically significant. However, Zn concentration decreased markedly from pH 6.07 to lower pHs. In both soils, non-rhizosphere soil had significantly higher Zn than rhizosphere soil in the higher pH

treatments. But the difference between non-rhizosphere and rhizosphere soils became insignificant at the two lowest pH treatments.

F3 Zn concentration increased with decreasing pH in the high metal soil (Fig. 3.26 a). And when pH >5.5, non-rhizosphere soil had more Zn than rhizosphere soil, at pH <5.5, this relationship was reversed. For low metal soil, F3 Zn concentrations showed a bell shape curve being the highest at the intermediate pH levels, with lower concentrations at both directions. Non-rhizosphere soil had higher Zn concentrations than rhizosphere soil (Fig. 3.26 b).

F4 Zn concentration declined with decreaing pH. The three higher pH treatments were significantly higher than the two lower pH treatments. Non-rhizosphere soil had slightly higher Zn concentrations than rhizosphere soil, but the difference was not significant (Fig. 3.27 a). For low metal soil, reduced pH also decreased the Zn concentration. From pH 7.27 to 6.07, Zn concentration changed only slightly while it was reduced significantly at lower pH treatments. When pH > 5.9, non-rhizosphere soil had more Zn than rhizosphere soil, however, when pH < 5.9, the reverse was true (Fig. 3.27 b).

The residual form of Zn was not significantly affected by reduced pH. Although concentrations tended to decrease at lower pH. For high metal soil, there was no significant difference between the four higher pH treatments. Only at the lowest pH treatment, was the Zn concentration significantly reduced (Fig. 3.28 a). For low metal soil, although there was a tendency of less Zn at lower pH there was no significant difference between all pH treatments (Fig. 3.28 b). For both high and low metal soils, there was no significant difference between non-rhizosphere soil and rhizosphere.

3.4 DISCUSSION

3.4.1 pH effects on metal extractability and distribution

Cd and Zn are present in many different forms in soil. Usually, dissolved hydrated metal ions in soil solution are the forms taken up by plant. In addition, soils contain a mixture of many colloidal organic and inorganic materials that can absorb and immobilize metals. Since different metal binding agents exhibit different response to the changes in soil equilibrium, it is essential to have a complete understanding of metal partitioning and distribution in soil.

In the present study, Cd and Zn were each partitioned into five fractions: soluble-exchangeable (F1), specifically sorbed-carbonated bound (F2), oxidizable (F3), reducible (F4), and residual (F5) forms. Prior to modification of pH, most Cd was in the second fraction-about 65% of the total Cd was present in this form in the high metal soil. The soluble form accounted for only about 3% of the total. There were low concentrations of Cd in pools F4 and F5. This is consistent with previous report that soil Cd is usually present in more labile pools (Ahnstrom and Parker, 2001; Hammer and Keller, 2002). Our data showed that reducing pH greatly altered Cd distribution among the five fractions. With decreasing pH, F1 was markedly increased, while F2 was equally decreased. For the high metal soil, from pH 6.88 to 4.74, F1 was increased from 0.81 to 6.41 mg kg^{-1}, while F2 was reduced from 15.93 to 9.70 mg kg^{-1}. About 16% of F3 Cd and 50% of F5 Cd became labile.

For the low metal soil, prior to reducing pH, about 52% of the total Cd was in F2, followed by F3, which accounted for 21% of total Cd. The soluble form (F1) accounted for only 7%. After reducing pH, at pH 4.74, most of the Cd was in F1, which now

accounted for about 50% of the total Cd. F2 decreased from 2.39 to 0.64 mg kg^{-1} and was only 17% of total Cd. F3 also decreased by 50% while F4 and F5 showed little change. The latter was not due to the lack of F4 and F5 becoming more labile, rather, it was because in the low metal soils, there was very little Cd in the F4 and F5 fractions to begin with. For both soils, increased soluble Cd was primarily from F2 or Cd retained by surface adsorption. If we combine F1 and F2, the sum was relatively constant at all pH treatments. This indicates that reducing pH primarily impacts F2, i.e., F2 is the fraction that most likely to change at reduced pH. Another important phenomenon is that total soil Cd changed with decreasing of pH. For high metal soil, it decreased from 24.7 to 22.7 mg kg^{-1} from the highest to the lowest pH. For the low metal soil, it changed from 4.6 to 3.9 mg kg^{-1}. This indicates as Cd became labile, it became easier to leach out of soil.

Prior to the pH treatment, most Zn was in the residual form (F5); about 36% of the total Zn was present in this form in the high metal soil, followed by F4, which counted for 33% of the total Zn; then F2, with about 20%. The soluble form contained the lowest Zn concentration, only 0.4%. With decreasing pH, F1 was markedly increased, while F2 was greatly decreased. From pH 6.88 to 4.74, F1 increased from 6 to 172 mg kg^{-1}, while F2 decreased from 342 to 187 mg kg^{-1}. About 45% of F2 Zn became more soluble. F3 decreased by 24% while F5 decreased by 11%. Interestingly, instead of a decrease, F3 increased from 33 to 50 mg kg^{-1} with decreasing pH. For the low metal soil, before reducing pH, about 46% of the total Zn was in the F5, followed by F4, which accounted for 35% of total Zn. The soluble form accounted for only 0.2% of the total. After reducing pH, at pH 4.74, F1 increased from 0.9 to 56 mg kg^{-1}, and contained 15% of the total Zn. However, F2 decreased from 37 to 13 mg kg^{-1}, F4 decreased from 164 to 102

mg kg^{-1}, and even F5 was reduced from 213 to 183 mg kg^{-1}. About 12% of total Zn was lost at the lowest pH treatment.

Very few studies have used complete sequential extraction procedures to link chemical reactivity with extractability. Ahnstrom and Parker (2001) used the isotope dilution method to investigate the relationship between the isotopic lability and chemical extractablility of Cd fractions. They used the same sequential extraction procedure as used here and found that in their Palmerton soil (total Cd concentration was similar to the high metal soil in this experiment), the percent of isotopic labile Cd were 70%, 41%, 3%, and 9% in F2, F3, F4, F5, respectively. The contribution of each fraction to the labile Cd pool was 14%, 50%, 35%, <1% and 1% for F1, F2, F3, F4, F5, respectively. They stated that the F4 and F5 fractions were dominated by nonlabile Cd. In this experiment, we also found that F4 was refractory. However, a large percent of F5 (50% in the high metal soil) became labile at sufficiently low pH. This high percentage may be related to the small size of F5 pool.

3.4.2 pH effect on *T. caerulescens* metal uptake

Lowering pH increased easily available Cd and Zn concentrations and enhanced metal uptake. On the other hand, low pH also increased some toxic elements, mostly Al and Mn in this experiment (Table 4.5), and restricted root development. Therefore, the highest plant tissue metal concentrations, as well as total metal translocated from soil were found at intermediate pH levels. This was what happened in the low metal soil. Metal concentration in the shoot biomass linearly increased with decreasing pH at the pH range 7.27-6.07 and reached the highest at pH 6.07. Concentrations then rapidly

decreased with further pH reduction. Plant growth was very poor at pH levels lower than 6.07 which illustrated Al toxicity (Fig. 3.1). However, for the high metal soil, results were quite different. Yield continued to increase and the highest yield was at the lowest pH treatment (Fig. 3.1). Few studies have investigated the relationship between pH and *T. caerulescens* metal uptake. In a greenhouse study, Brown et al. (1994) used three soils adjusted to three different pH levels. Total translocated Zn was highest at the highest pH (6.82) in one soil and in another two soils it was at the pH of 5.42 and 6.67, the intermediate pH values among the three levels. Total translocated Cd was highest at the highest pH (6.37 and 7.04) in two soils and in another soil it was at the intermediate pH of 5.81.

In a field study, soil pH had no effect on Zn uptake, but lowering pH increased Cd uptake at the two highest metal treatments. In the control and low metal treatments, there was no significant difference in uptake (Brown et al., 1995 b). A possible reason for the lack of difference is that lowering pH affected both plant growth and metal uptake. In the greenhouse study, the negative effect on reduction of yield of lowering pH was dominant, while in the field study, the metal concentration was too low to observe a strong pH effect.

Kayser et al. (2000) used sulfur to reduce soil pH and observed a more consistent effect of enhancing Zn and Cd uptake by other plant species, *B. juncea, N. tabacum, S. Viminalis, H. annuus, Z. mays.* But *T. caerulescens* was too sensitive to low pH to survive in this experiment. Sulfur caused a small decrease in soil pH but a significant increase in Zn and Cd mobility. These authors therefore attributed the S effect to soluble salts rather than a direct pH influence.

3.4.3 Relationship between metal bioavailability and plant uptake

Researchers have unsuccessfully tried to correlate soil metal concentrations with plant concentrations using different extraction methods (Tsadilas et al., 1995; Sims and Kline, 1991). Although SEP is useful as an indicator of metal bioavailability, correlation studies are usually of limited use in interpreting bioavailability. As stated by Giller et al. (1998) “What is meant by ‘bioavailability’ is usually ill-defined and is rarely quantified”, “In reality, bioavailability cannot be measured, because it can only be assessed by the growth of the organism of interest and an evaluation of the uptake or toxicity of a metal after the fact” (Wolt, 1994).

This statement was supported by our Cd data. Using rhizosphere soil metal concentrations to correlate *T. caerulescens* tissue metal concentration with Cd in each fraction, there were no significant correlations between shoot Cd concentration with F1 ($r = 0.03$, $p = 0.86$). Whereas, shoot Cd was highly correlated with all other fractions. However, when we correlated non-rhizosphere soil metal concentrations with uptake, results were quite different. In non-rhizosphere soil, F1 was significantly correlated with shoot Cd ($r = 0.53$, $p<0.001$), as well as other fractions. The most highly correlated fractions were F2 ($r = 0.89$, $p<0.0001$) and F3 ($r = 0.93$, $p<0.0001$). Comparing total Cd in the plant shoot and Cd F1 pool (except pH 4.74), total Cd extracted was smaller than the F1 Cd pool. *T. caerulescens* must have used Cd from other non-labile pools. In these treatments, uptake by *T. caerulescens* is limited by the amount of “direct available” metal ions and must rely on soils replenishing ability and high soil surface area. Therefore the original F1 pool is the available metal content at soil equilibrium and the larger it was, the higher was the soils ability to replenish Cd. If the original F1 metal pool is larger than

total metals plants have taken up, plant uptake will not rely on soils replenishment ability. On the contrary, if the original F1 metal pool is smaller than total metals plants have taken up, plant uptake will be limited by soils replenish ability. Based on this analysis, if we remove the treatments that F1 pool exceeded all metal plant taken up, F1 should be a better indicator of metal "bioavailability". After we remove pH 4.74 treatment from the high metal soil, and pH 4.74 and 5.28 treatments from the low metal soil, the correlation coefficient of shoot Cd concentration and CdF1 increased to 0.82 ($p<0.0001$). Similarly, when correlataing rhizosphere Zn concentrations with plant Zn concentration, shoot Zn was not correlated with F1 ($r = -0.05$, $p = 0.77$) while significantly correlated with all the other fractions. However, after removal of the treatments where the F1 pool exceeded plant total uptake, shoot Zn concentration was most significantly correlated with F1 ($r=0.45$, $p=0.01$), and secondly correlated with F3. There was no correlation with F2, F4, or F5. This showed that care must be exercised when interpreting metal "bioavailability" in correlation studies. A valid connection only happened when metal concentration being used was a "before-fact" concentration and metal bioavailabilitywa s a limiting factor for plant uptake and the replenishing mechanisms, either through soil buffering capacity or plant solubilization are not sufficient for plant uptake, i.e., plant is constantly under the pressure of metal limitation.

3.4.4 Effect of *T. caerulescens* on Cd and Zn distribution-Changes in the rhizosphere soil metal environment

In the high metal soil, *T. caerulescens* reduced total Cd by 19% to 37%. Cd in pools of F1, F2, and F3 were most significantly affected. *Thlaspi caerulescens* nearly depleted

Cd in the F1 pool while it also significantly reduced the amount of Cd in the pools of F2 and F3 at all pH treatments ($p<0.01$). *Thlaspi caerulescens* was also able to reduce Cd in the pools of F4 and F5 at all pH treatments, but to a lesser extent. For F4, the reduction was significant at pH of 4.74, 5.28, and 6.37 ($p<0.05$). For the F5 pool, the reduction was significant at pH treatment of 6.07 and 6.37 ($p<0.05$). In the case of low metal soil, *T. caerulescens* reduced total Cd by 5% to 45%. The effect of *T. caerulescens* on F1, F2, F3 pools was similar to the high metal soil. But for F4 and F5, since there was only marginal Cd, and in most cases below the detection limits, no changes were noted.

In the high metal soil, *T. caerulescens* reduced the total Zn by 1% to 2%. Zn in pools of F1 and F2 was most significantly affected. *Thlaspi caerulescens* significantly reduced the amount of Zn in F1 pool in all pH treatments and F2 pool at pH treatments of 6.37 and 6.88 ($p<0.05$). *Thlaspi caerulescens* did not cause significant changes in the F3, F4 and F5 pools. For low metal soil, *T. caerulescens* reduced the total Zn by 3% to 8%. *Thlaspi caerulescens* significantly reduced the amount of Zn in the F1 pool in the treatments of 6.07, 5.28, and 4.74 and F2 pools at pH treatments of 6.07, 6.37, 6.88 and 7.27 ($p<0.05$). *Thlaspi caerulescens* could also access the F3 pool; Zn in this fraction was also reduced at all pH treatments. But the reduction was only significant at pH 6.07 and 6.37. *Thlaspi caerulescens* did not cause significant changes in the F4 and F5 pools.

The preference for specific metal pools of Cd has also been observed by Hammer and Keller (2002) using a different sequential extraction procedure. However, they did not observe changes for Zn. Indeed, southern France genotype of *T. caerulescens* altered Cd to a much greater extent than Zn. Total Cd was reduced 37% and 45% in the high and low metal soils, respectively, after only one planting, indicating rapid remediation of Cd.

A study where phytoextration of three continuous crops of *T. caerulescens* were investigated indicated that Zn concentrations in *T. caerulescens* tissue were relatively constant in one soil and increased in subsequent croppings in the other soil while cadmium concentrations did not change for one soil and were unchanged in the first and third croppings while significantly increased in the second cropping in the other soil (Keller and Hammer, 2004). However, complete sequential extraction combined with continuous cropping is still needed to assess the possible changes in metal distribution in subsequent croppings. Our data also show that due to the ability of soils to replenish specific pools, *T. caerulescens* uptake was not exclusively confined by original available forms of metals. Similarly, other studies have found that for *T. caerulescens*, metals released from formerly non-available forms could reach more than 50% of the metals accumulated in plants (Knight et al., 1997 a; Whiting et al., 2001a; Whiting et al., 2001 b). In other words, depletion of soluble metal pool is not “definitive”. Soils can rapidly replenish and reach a new equilibrium. Therefore, significant reduction in total metal concentration is more relevant since this will force soil to have lower bioavailable metals even under new equilibrium at the same environmental conditions.

In conclusion, reducing pH is an effective method to enhance metal bioavailability and *T. caerulescens* uptake for both Cd and Zn. However, the proper and effective pH range for maximum metal uptake may differ for individual soils.

3.5 CONCLUSIONS

These results indicate that:

1) Reducing pH significantly redistributed Cd and Zn among five fractions. The soluble metal form (F1) was greatly increased; F2, F3, F4, and F5 all had different degree of mobilization under low pH.
2) Reducing pH significantly influenced plant metal uptake. For the high metal soil, plants grew best at the lowest pH treatment and the highest metal concentration was at the second lowest pH treatment. For low metal soil, due to low pH induced Al and Mn toxicity, both plant growth and metal uptake were the best at intermediate pH level.
3) Plant uptake of metals significantly modified the rhizosphere soil metal environment. *Thlaspi caerulescens* was able to reduce Cd concentration in all 5 fractions, where F1, F2, and F3 were most significantly affected. For Zn, *T. caerulescens* significantly reduced metal concentrations in the F1 and F2 pools and caused no significant changes in the F3, F4, and F5 pools.

Table 3.1. Summary of sequential extraction procedure

Fraction	Operational definition	Extractant	#of treatment	Operation	Reference
F1	Soluble-exchangeable	15 ml 0.1 M $Sr(NO)_3$	2	Shake 2h	Ahnstrom and Parker (1999)
F2	Sorbed-carbonate	15 ml 1.0 M NaOAc, pH5.0	2	Shake 5h	Ahnstrom and Parker (1999)
F3	Oxidizable	5 ml 5% NaOCl, pH 8.5	3	95^{o}C water bath 30min	Ahnstrom and Parker (1999)
F4	Reducible	20 ml 0.4 M oxalate +0.1 ascorbate, pH 3.0	3	95^{o}C water bath 30min	Ahnstrom and Parker (1999)
F5	Residual	Aqua regia	1	hot plate	McGrath and Cunliffe (1985)

Table 3.2. Soil Properties

Soil	Total Zn	Total Cd	Texture	pH	O.M.	CEC	Sand	Silt	Clay	M (I) – P	M (I) – K	N
	mg kg^{-1}	mg kg^{-1}			%	cmol kg^{-1}	%	%	%	mg kg^{-1}	mg kg^{-1}	%
Low metal	450	5.0	loam	7.3	4.7	29.5	36.5	38.0	25.5	68.4	249	0.075
High metal	1500	25.4	loam	6.9	5.2	11.2	39.5	34.5	26.0	265	295	0.096

Table 3.3. Summary of analysis of variance of the effect of pH and metal concentration in *T. caerulescens* biomass

ANOVA Source of variation	df	Shoot yield	Shoot Cd conc.	Shoot Zn conc.	Root Cd conc.	Root Zn conc.	Total shoot Cd accumulated	Total shoot Zn accumulated
		---------- F value ----------						
pH	4	2.20	55.6***	42.1***	0.72	1.74	5.35**	4.31**
Metal (M)	1	32.3***	1854***	71.8***	67.6***	9.84**	519***	41.2***
pH x M	4	11.5***	63.0***	48.2***	1.25	5.69**	18.4***	22.0***

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 3.4. Summary of analysis of variance of the effect of pH and soil metal concentration on the concentration of other elements and heavy metals in *T. caerulescens* shoot and root tissues.

ANOVA		Shoot							Root						
Source of variation	df	Ca	Cu	Fe	Mg	Mn	K	P	Ca	Cu	Fe	Mg	Mn	K	P
		F value													
pH	4	14.2***	8.2***	3.4*	3.0*	503***	3.7*	1.3	0.78	2.0	0.78	10.8***	48.4***	1.6	2.81*
Metal (M)	1	16.0***	9.1**	5.3*	170***	425***	0.65	0.43	4.3*	55.0***	8.42**	90.5***	4.7*	2.8	18.7***
pH x M	4	18.5***	4.9**	2.6	5.6**	41.4***	0.50	4.5**	2.5	1.28	1.82	0.93	8.6***	5.3**	4.7**

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 3.5. Summary of analysis of variance of the effect of pH, location, and metal concentration on the distribution of Cd in five sequential extraction fractions

ANOVA Source of variation	df	F1	F2	F3	F4	F5
		F value				
pH	4	551***	284***	53***	< 1	6***
Location(L)	1	1223***	2113***	794***	2	2
Metal (M)	1	429***	11984***	6997***	217***	152***
pH x L	4	90***	17***	5**	< 1	<1
pH x M	4	6***	25***	14***	< 1	6***
L x M	1	292***	< 1	415***	5*	2
pH x L x M	4	5**	95***	13***	< 1	<1

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 3.6. Summary of analysis of variance of the effect of pH, location, and metal concentration on the distribution of Zn in five sequential extraction fractions

ANOVA Source of variation	df	F1	F2	F3	F4	F5
		---------- F value ----------				
pH	4	1506***	236***	2	2	1
Location(L)	1	182***	71***	7*	< 1	< 1
Metal (M)	1	1119***	17404***	988***	196***	493***
pH x L	4	19***	11***	< 1	< 1	< 1
pH x M	4	77***	22***	15***	< 1	< 1
L x M	1	< 1	26***	3**	< 1	< 1
pH x L x M	4	17***	5**	< 1	< 1	< 1

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 3.7. Pearson correlation coefficients between pH and shoot element concentrations.
N = 88 Prob > |r| under H0: Rho=0

	pH	Zn	Cd	Mn	Fe	Ca	Mg
Zn	0.03ns						
Cd	-0.17ns	0.61 ***					
Mn	-0.70***	-0.45**	-0.38 *				
Fe	-0.33*	-0.16ns	-0.24ns	0.58***			
Ca	0.55***	0.12ns	-0.20ns	-0.48***	-0.27ns		
Mg	-0.28ns	0.43**	0.79***	-0.08ns	-0.01ns	-0.49***	
Cu	-0.52***	-0.20ns	-0.21ns	0.74***	0.54***	-0.44**	0.05ns

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 3.8. Regression models and significant levels for the effect of pH on soil Cd distribution in five sequential extraction fractions of the high metal soil

Fraction	Soil type	Regression equation	R square
F1	Non-rhizo.	$\log Cd = 16.1 - 4.39 \times pH + 0.29 \times pH^2$	0.99***
	Rhizo.	$\log Cd = 15.6 - 5.21 \times pH + 0.41 \times pH^2$	0.68***
F2	Non-rhizo.	$\log Cd = -4.15 + 2.15 \times pH - 0.17 \times pH^2$	0.93***
	Rhizo.	$\log Cd = -10.2 + 3.59 \times pH - 0.26 \times pH^2$	0.78***
F3	Non-rhizo.	$Cd = -9.57 + 4.66 \times pH - 0.38 \times pH^2$	0.72***
	Rhizo.	$Cd = -12.8 + 4.78 \times pH - 0.35 \times pH^2$	0.78***
F4	Non-rhizo.	non significant	
	Rhizo	$\log Cd = -4.45 + 1.35 \times pH - 0.11 \times pH^2$	0.94***
F5	Non-rhizo.	$\log Cd = -0.72 + 0.35 \times pH$	0.92***
	Rhizo.	$\log Cd = -0.72 + 0.32 \times pH$	0.93***

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 3.9. Regression models and significant levels for the effect of pH on soil Cd distribution in five sequential extraction fractions in the low metal soil

Fraction	Soil type	Regression equation	R square
F1	Non-rhizo.	$\log Cd = 13.5 - 3.90 \times pH + 0.26 \times pH^2$	0.91***
	Rhizo.	$\log Cd = 15.3 - 5.08 \times pH + 0.39 \times pH^2$	0.85***
F2	Non-rhizo.	$\log Cd = -13.8 + 2.15 \times pH - 0.31 \times pH^2$	0.99***
	Rhizo.	$\log Cd = 6.59 - 2.73 \times pH + 0.25 \times pH^2$	0.69***
F3	Non-rhizo.	$Cd = -4.83 + 1.68 \times pH - 0.12 \times pH^2$	0.97***
	Rhizo.	$Cd = -0.19 + 0.11 \times pH$	0.53***
F4	Non-rhizo.	non significant	
	Rhizo	non significant	
F5	Non-rhizo.	non significant	
	Rhizo.	non significant	

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 3.10. Regression models and significant levels for the effect of pH on soil Zn distribution in five sequential extraction fractions in the high metal soil

Fraction	Soil type	Regression equation	R square
F1	Non-rhizo.	$\log Zn=27.4 - 6.85\times pH + 0.46\times pH^2$	0.99***
	Rhizo.	$\log Zn=31.6 - 8.49\times pH + 0.60\times pH^2$	0.98***
F2	Non-rhizo.	$\log Zn= -3.57 + 2.98\times pH - 0.24\times pH^2$	0.91***
	Rhizo.	$\log Zn= -1.63 + 2.35\times pH - 0.19\times pH^2$	0.91***
F3	Non-rhizo.	$\log Zn=4.81 - 0.20\times pH$	0.91***
	Rhizo.	$\log Zn=4.99 - 0.23\times pH$	0.90***
F4	Non-rhizo.	$\log Zn=5.42 + 0.11\times pH$	0.98***
	Rhizo	$\log Zn=5.40 + 0.11\times pH$	0.99***
F5	Non-rhizo.	$\log Zn= 4.40 + 0.71\times pH - 0.06\times pH^2$	0.96***
	Rhizo.	$\log Zn=4.80 + 0.59\times pH - 0.05\times pH^2$	0.98***

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 3.11. Regression models and significant levels for the effect of pH on soil Zn distribution in five sequential extraction fractions in the low metal soil

Fraction	Soil type	Regression equation	R square
F1	Non-rhizo.	$\log Zn = 13.4 - 1.93 \times pH$	0.89***
	Rhizo.	$\log Zn = 37.2 - 10.4 \times pH + 0.72 \times pH^2$	0.91***
F2	Non-rhizo.	$\log Zn = -11.4 + 4.61 \times pH - 0.35 \times pH^2$	0.97***
	Rhizo.	$\log Zn = -3.85 + 2.07 \times pH - 0.15 \times pH^2$	0.82***
F3	Non-rhizo.	$\log Zn = -8.61 + 3.61 \times pH - 0.29 \times pH^2$	0.96***
	Rhizo.	$\log Zn = -4.05 + 2.11 \times pH - 0.17 \times pH^2$	0.84***
F4	Non-rhizo.	$\log Zn = 3.65 + 0.20 \times pH$	0.93***
	Rhizo	$\log Zn = 3.90 + 0.15 \times pH$	0.94***
F5	Non-rhizo.	$\log Zn = 4.00 + 0.44 \times pH - 0.03 \times pH^2$	0.96***
	Rhizo.	$\log Zn = 4.59 + 0.11 \times pH$	0.18*

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 3.12. Pearson correlation coefficients between Cd and Zn fractions using non-rhizosphere soil metal concentration data Prob > |r| under H0: Rho=0

	pH	shootZn	rootZn	CdF1	CdF2	CdF3	CdF4	CdF5
pH	1.00	0.03ns	-0.42**	-0.74***	0.07ns	-0.03ns	-0.17ns	0.13ns
ShootCd	-0.17ns	0.61***	0.49***	0.52***	0.89***	0.93***	0.73***	0.68***
RootCd	-0.25ns	0.31*	0.62***	0.59***	0.70***	0.76***	0.71***	0.48**
ZnF1	-0.77***	0.03ns	0.59***	0.98***	0.08ns	0.22ns	0.30*	-0.06ns
ZnF2	0.06ns	0.45**	0.29ns	0.22ns	1.00***	0.98***	0.72***	0.86***
ZnF3	-0.25ns	0.50***	0.52***	0.63***	0.84***	0.91***	0.86***	0.56***
ZnF4	0.01ns	0.38*	0.25ns	0.28ns	0.80***	0.82***	0.92***	0.49***
ZnF5	-0.06ns	0.41**	0.42**	0.36*	0.90***	0.90***	0.50***	0.91***

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

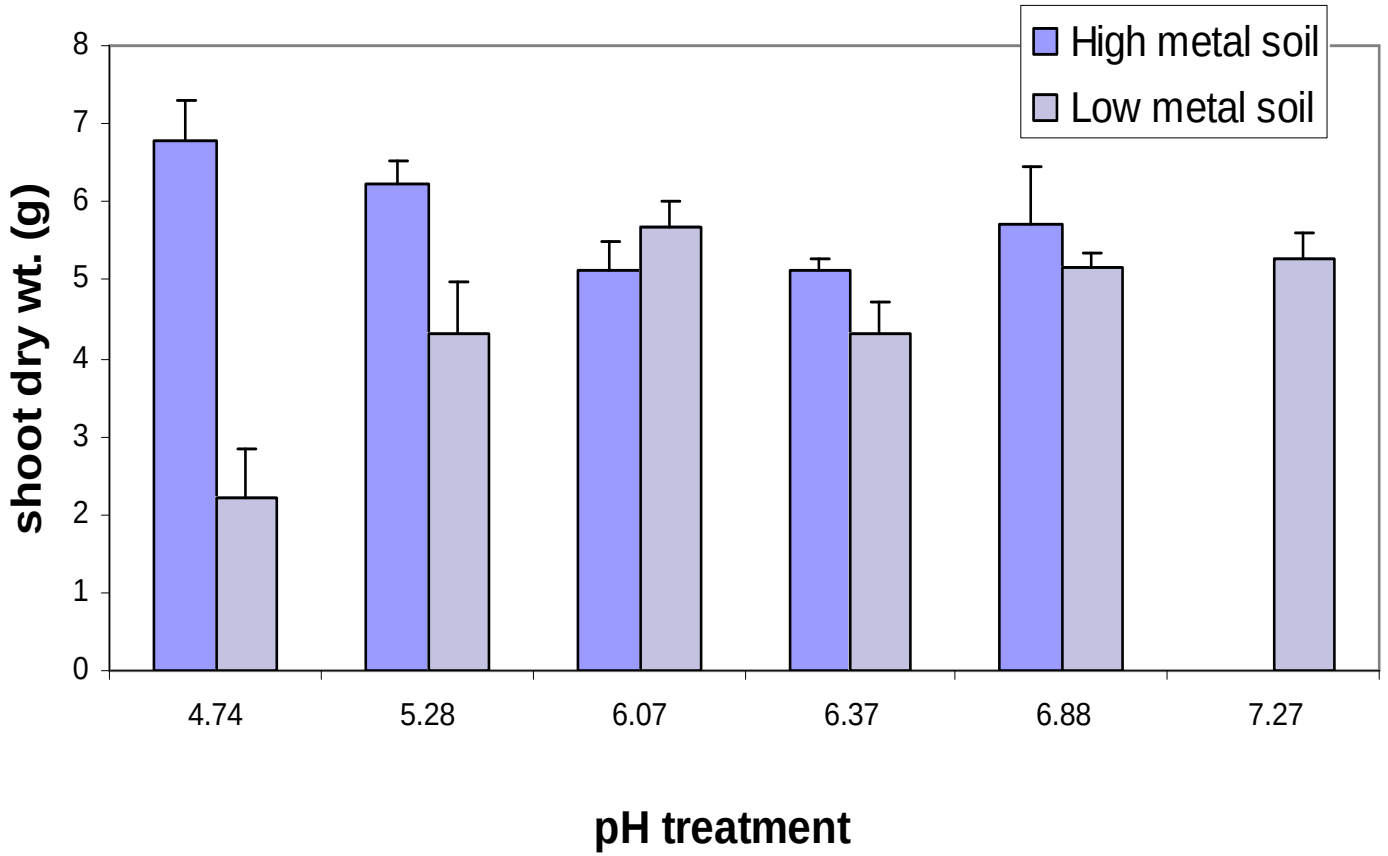

Figure 3.1. Means and standard errors of *Thlaspi caerulescens* shoot dry weight with different pH treatments.

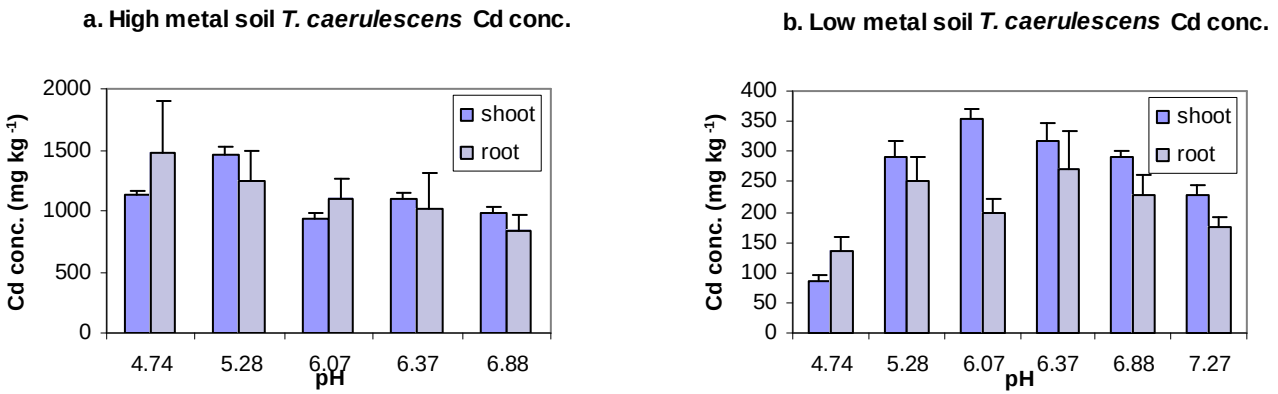

Figure 3.2. Means and standard errors of *T. caerulescens* tissue Cd concentration in high metal soil (a) and low metal soil (b) with different pH treatments.

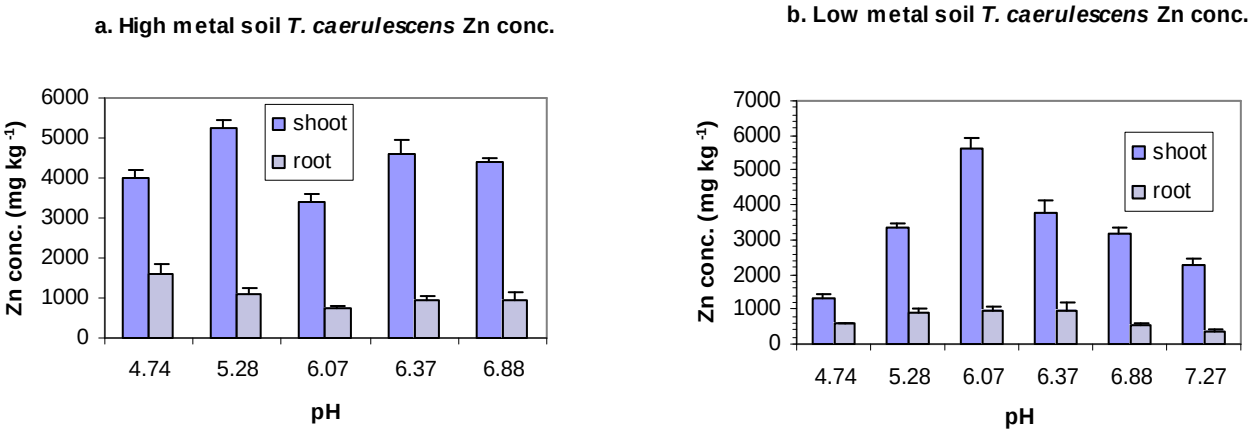

Figure 3.3. Means and standard errors of *T. caerulescens* tissue Zn concentration in high metal soil (a) and low metal soil (b) with different pH treatments.

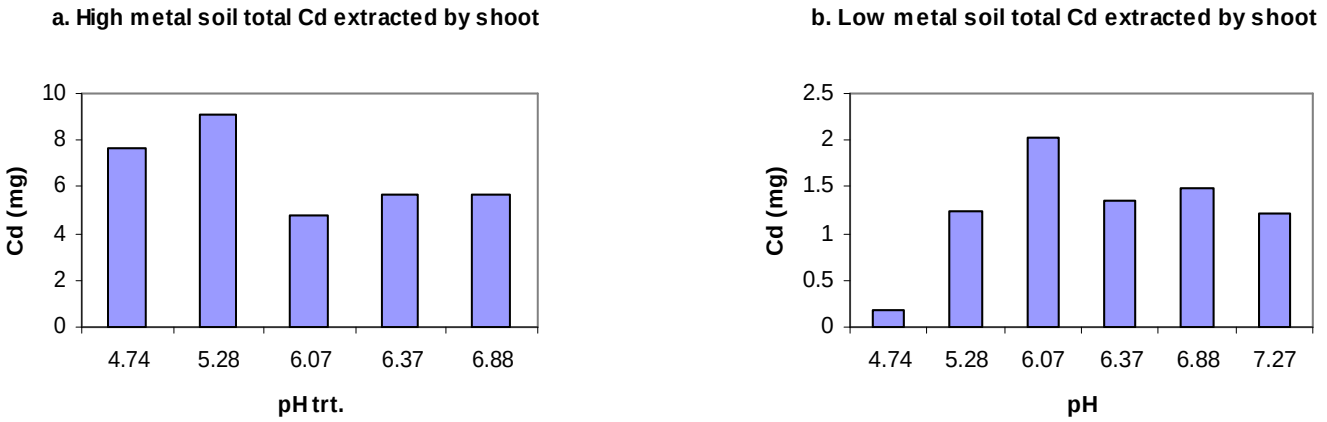

Figure 3.4. Means of total Cd phytoextracted to shoot biomass of *T. caerulescens* in the high metal soil (a) and low metal soil (b).

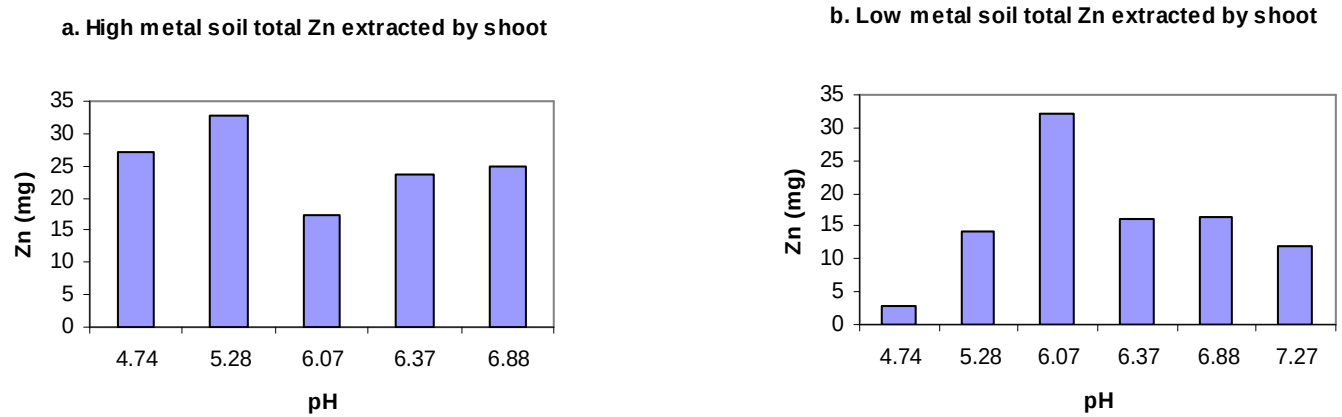

Figure 3.5. Means of total Zn phytoextracted to shoot biomass of *T. caerulescens* in the high metal soil (a) and low metal soil (b).

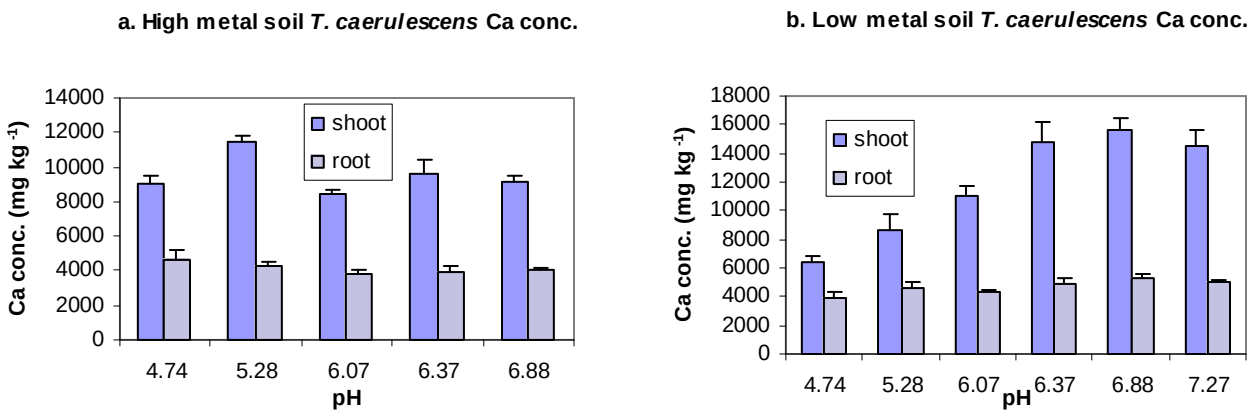

Figure 3.6. Means and standard errors of *T. caerulescens* tissue Ca concentration in high metal soil (a) and low metal soil (b) with different pH treatments.

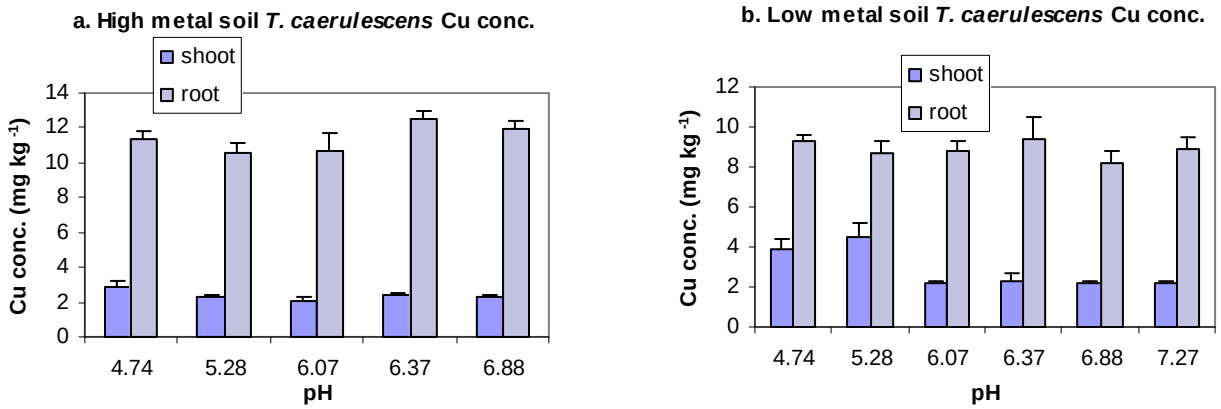

Figure 3.7. Means and standard errors of *T. caerulescens* tissue Cu concentration in high metal soil (a) and low metal soil (b) with different pH treatments.

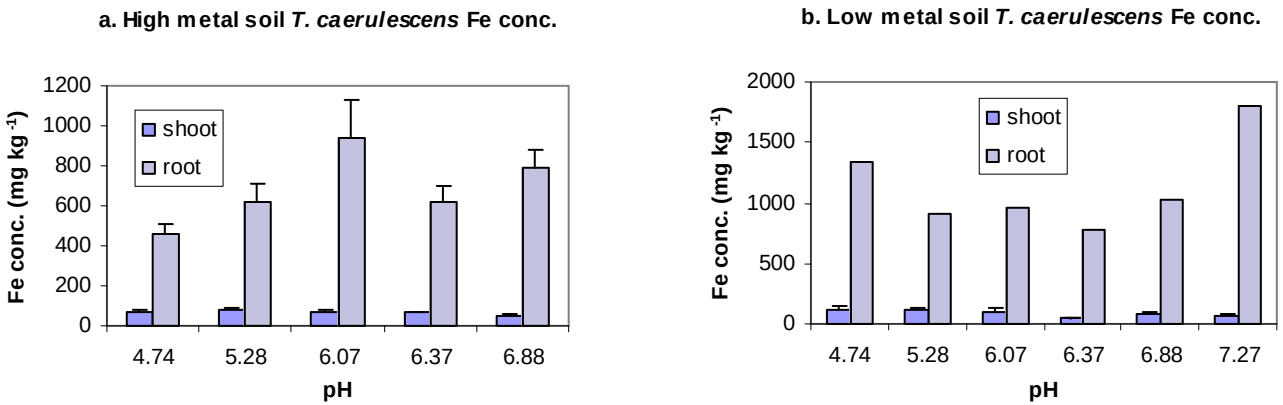

Figure 3.8. Means and standard errors of *T. caerulescens* tissue Fe concentration in high metal soil (a) and low metal soil (b) with different pH treatments.

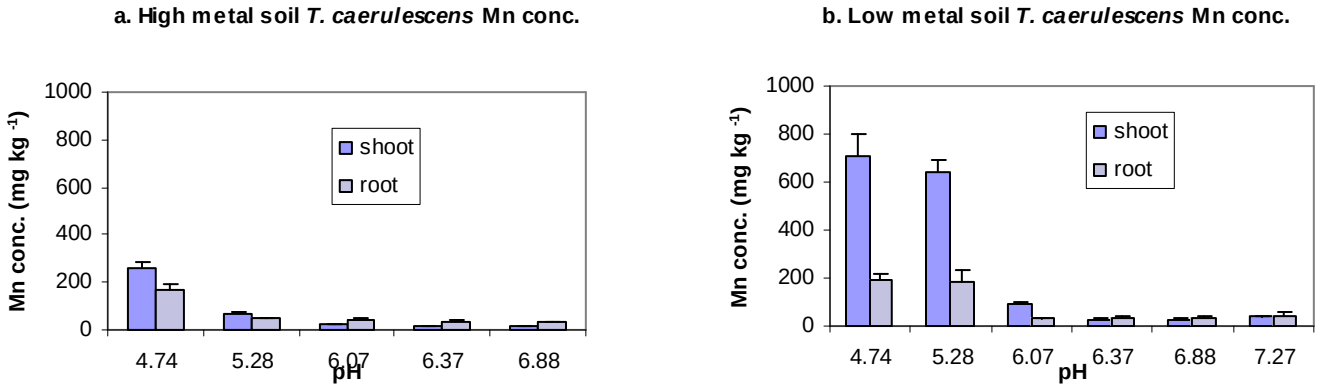

Figure 3.9. Means and standard errors of *T. caerulescens* tissue Mn concentration in high metal soil (a) and low metal soil (b) with different pH treatments.

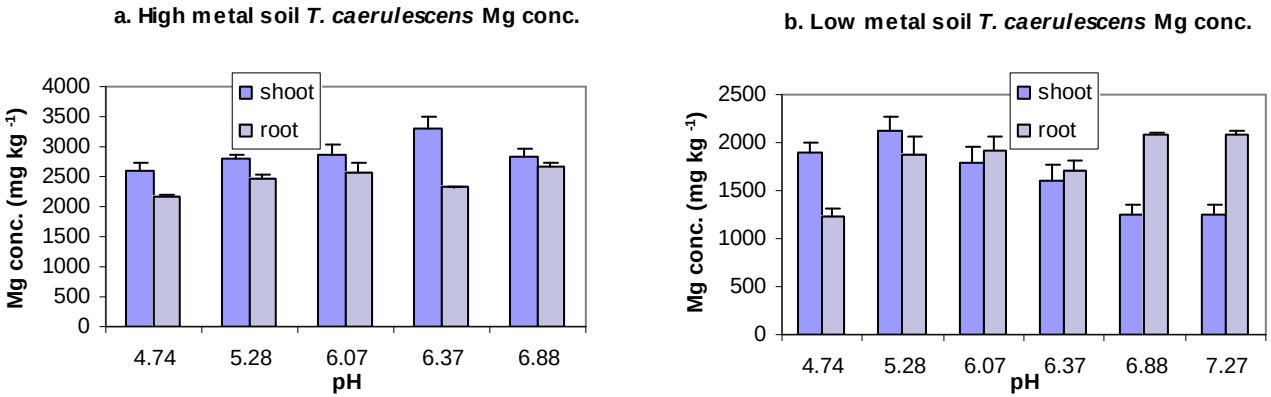

Figure 3.10. Means and standard errors of *T. caerulescens* tissue Mg concentration in high metal soil (a) and low metal soil (b) with different pH treatments.

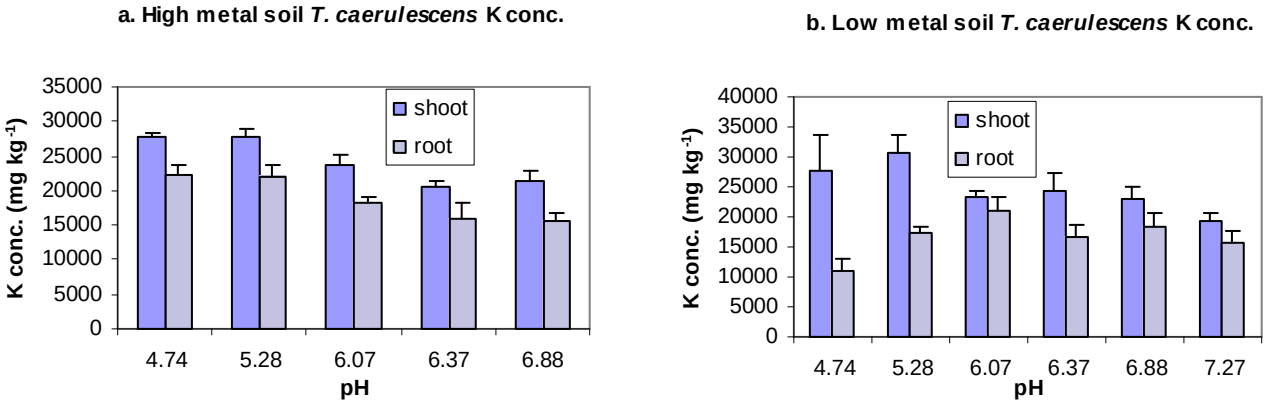

Figure 3.11. Means and standard errors of *T. caerulescens* tissue K concentration in high metal soil (a) and low metal soil (b) with different pH treatments.

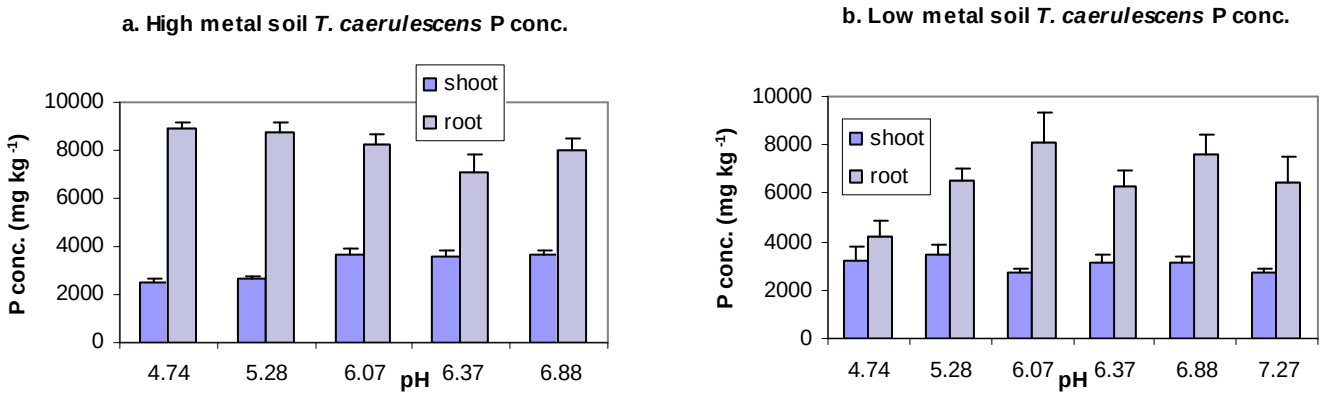

Figure 3.12. Means and standard errors of *T. caerulescens* tissue P concentration in high metal soil (a) and low metal soil (b) with different pH treatments.

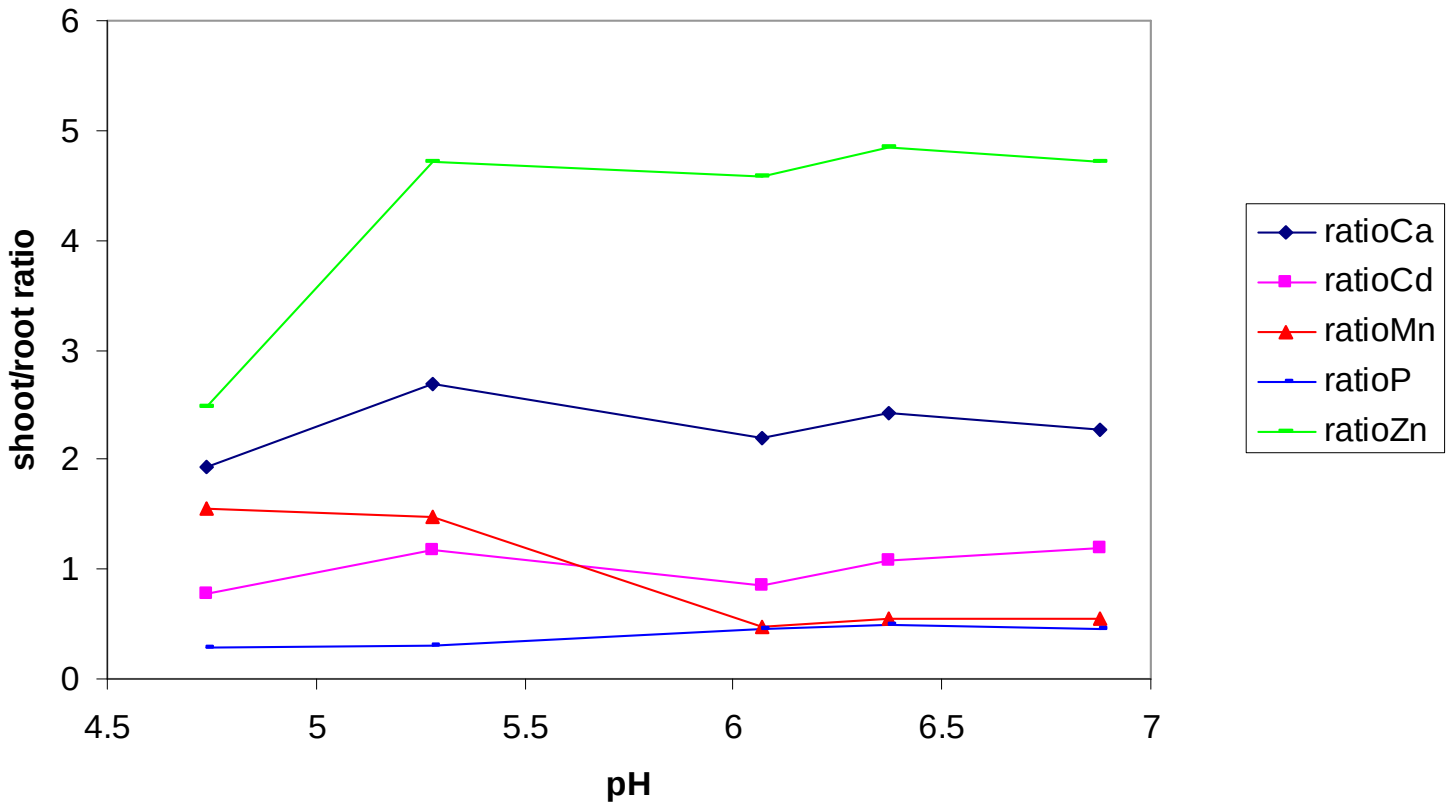

Figure 3.13. Relationship of shoot/root concentration ratio with pH in high metal soil. Elements of K, Mg, Cu and Fe showed little variability and were omitted from this graph.

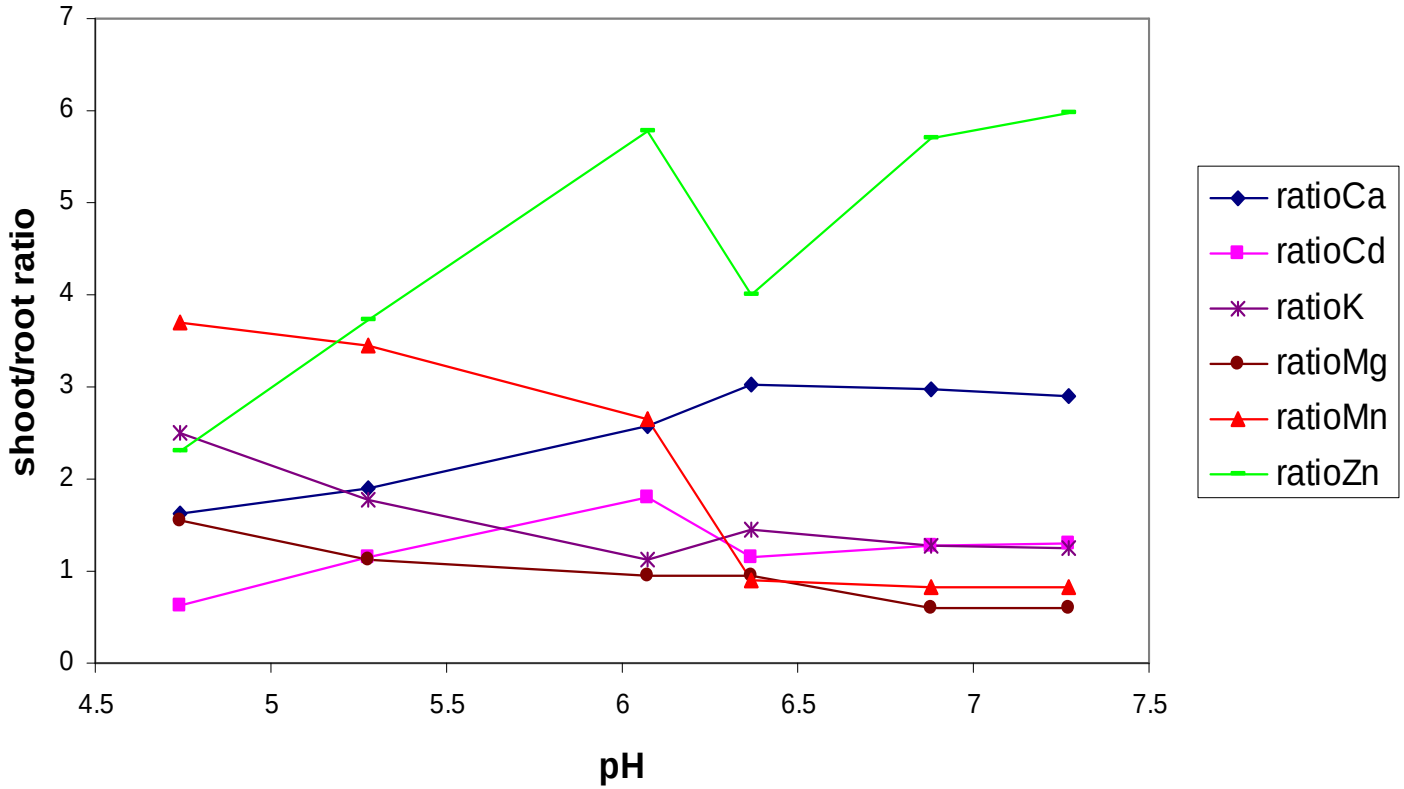

Figure 3.14. Relationship of shoot/root concentration ratio with pH in low metal soil. Elements of Cu and Fe showed little variability and were omitted from this graph.

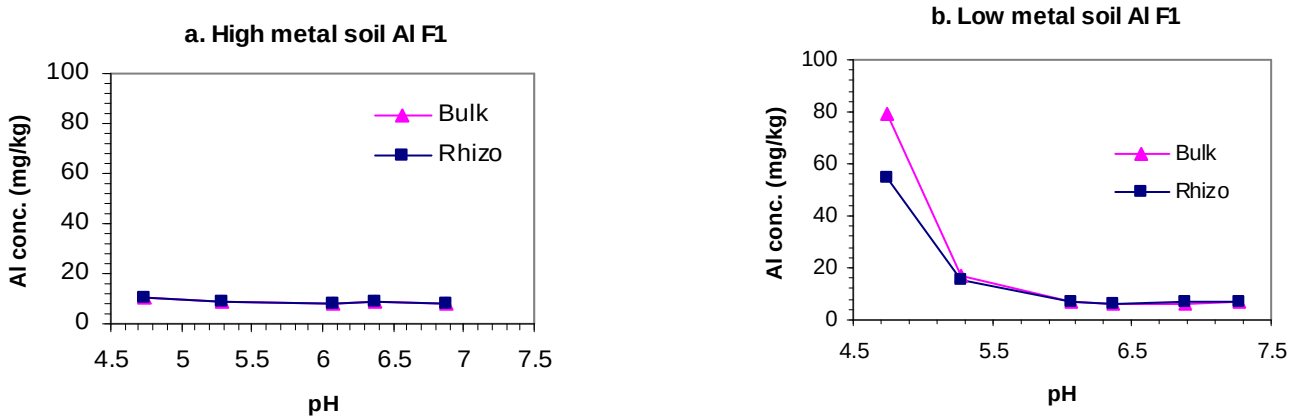

Figure 3.15. 0.1 M $Sr(NO_3)_2$ extractable Al concentrations in high metal soil (a) and low metal soil (b) with difffent pH treatments.

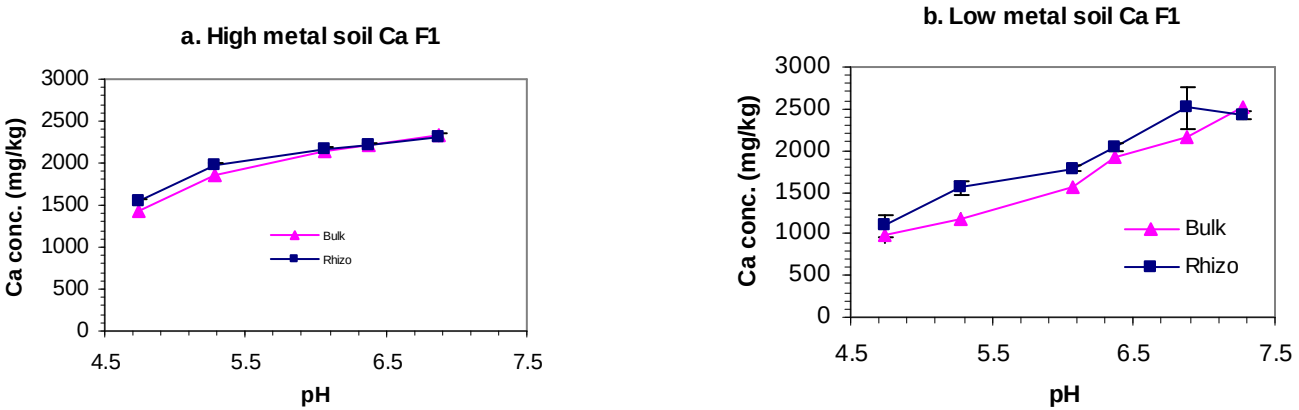

Figure 3.16. 0.1 M $Sr(NO_3)_2$ extractable Ca concentrations in high metal soil (a) and low metal soil (b) with different pH treatments.

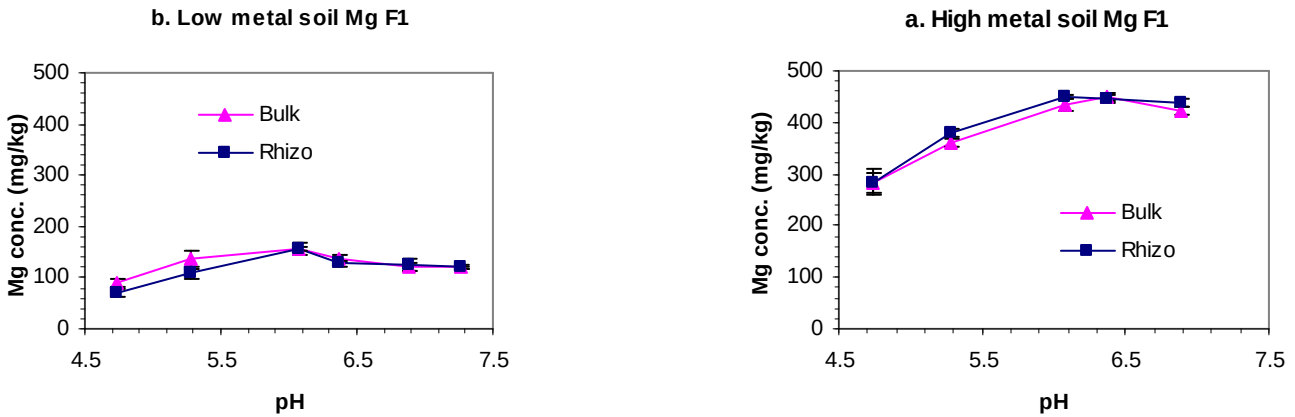

Figure 3.17. 0.1 M $Sr(NO_3)_2$ extractable Mg concentrations in high metal soil (a) and low metal soil (b) with different pH treatments.

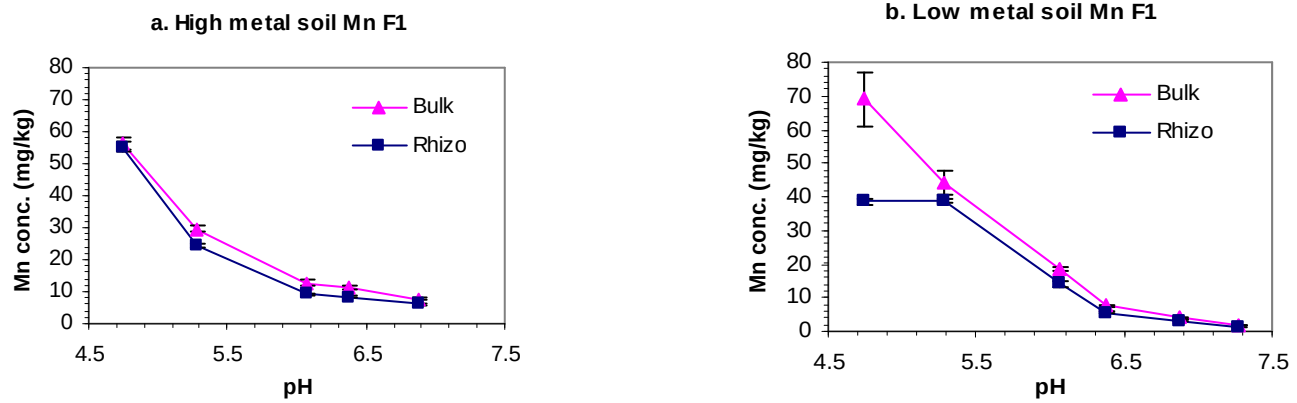

Figure 3.18. 0.1 M $Sr(NO_3)_2$ extractable Mn concentrations in high metal soil (a) and low metal soil (b) with different pH treatments.

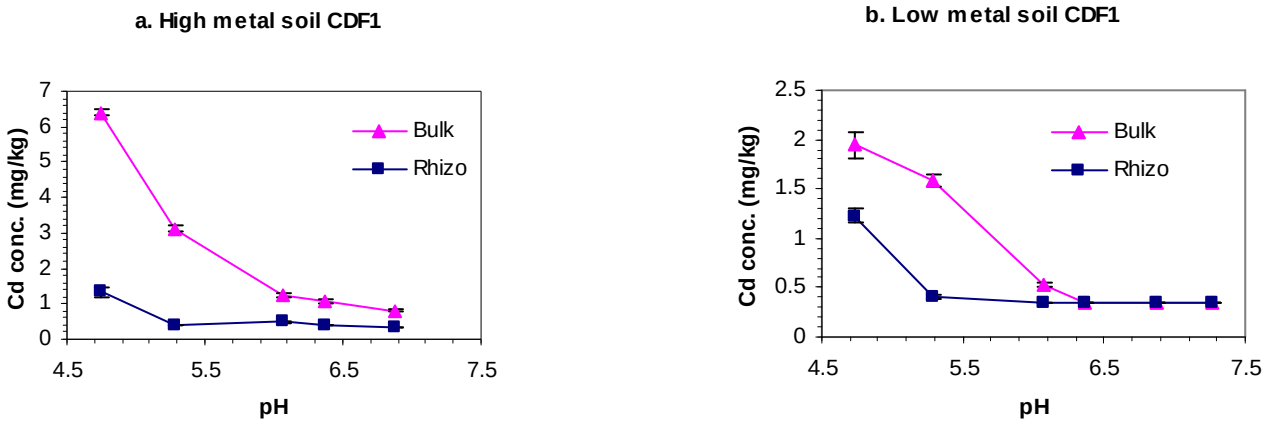

Figure 3.19. 0.1 M $Sr(NO_3)_2$ extractable Cd concentrations in high metal soil (a) and low metal soil (b) with different pH treatments.

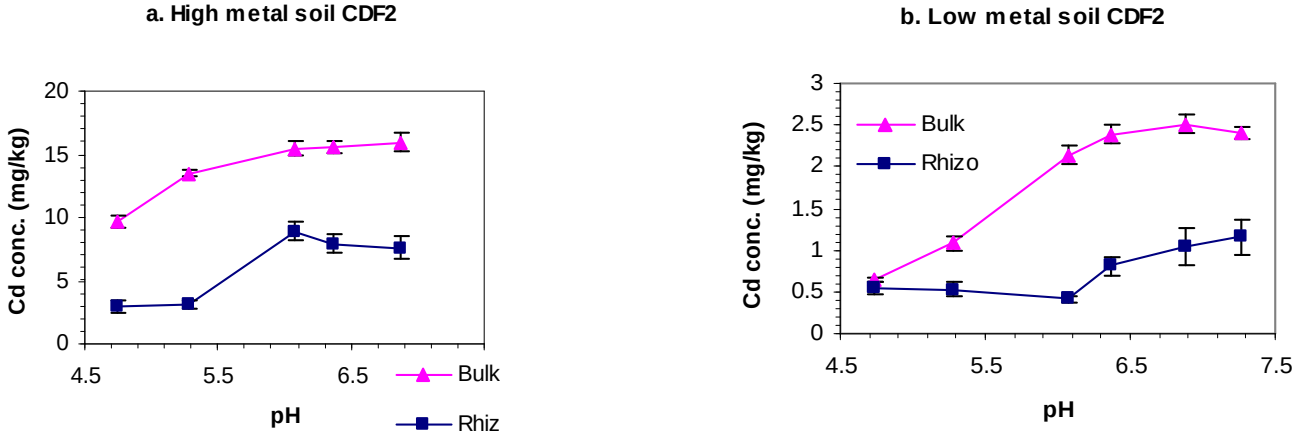

Figure 3.20. 1.0M NaOAc pH 5.0 extractable Cd concentrations in high metal soil (a) and low metal soil (b) with different pH treatments.

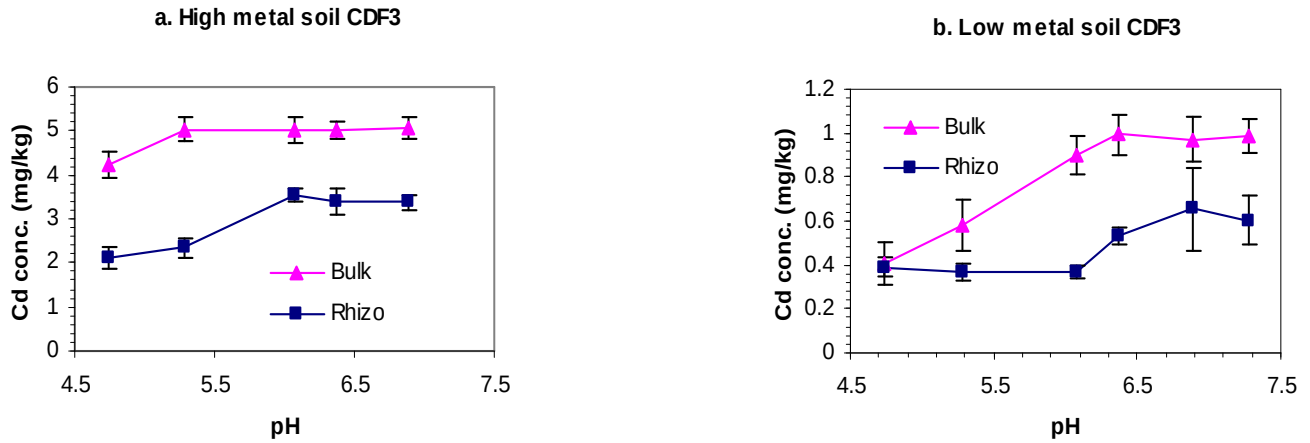

Figure 3.21. 5% NaOCl pH 8.5 extractable Cd concentrations in high metal soil (a) and low metal soil (b) with different pH treatments.

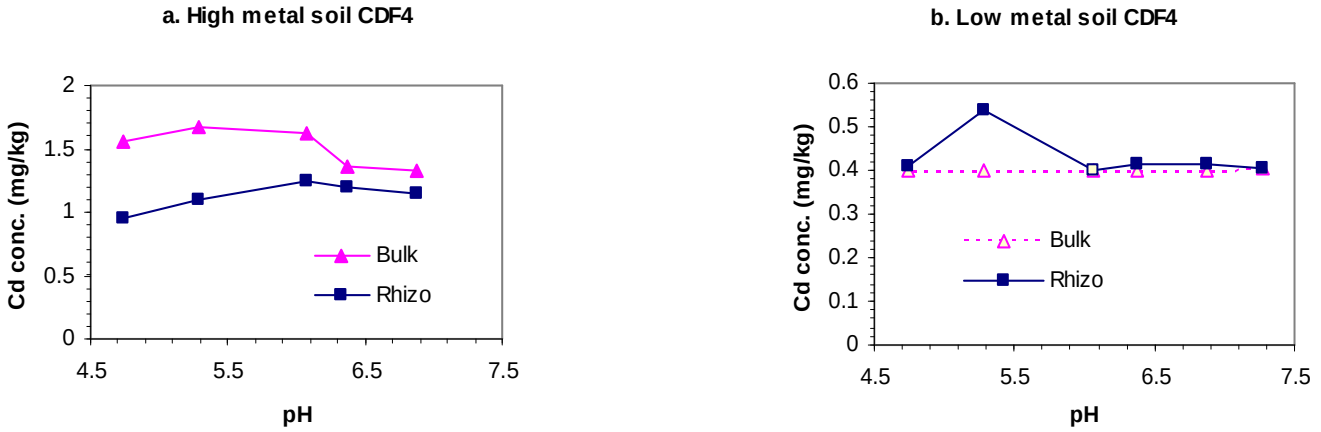

Figure 3.22. 0.4 M oxalate plus 0.1 M ascorbate pH 3.0 extractable Cd concentrations in high metal soil (a) and low metal soil (b) with different pH treatments. Blank symbols and dotted line indicate belowing detection limit.

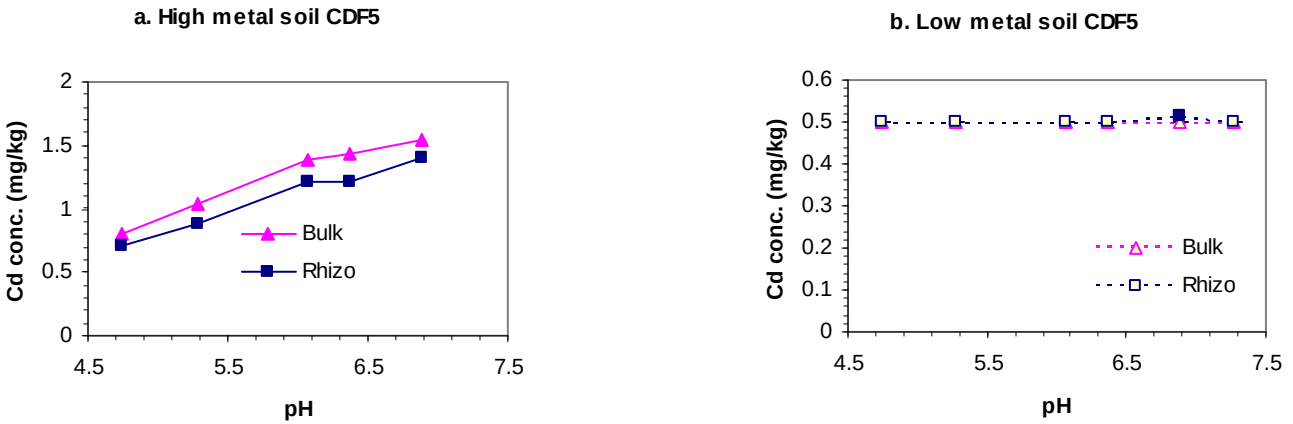

Figure 3.23. Residual Cd concentrations in high metal soil (a) and low metal soil (b) with different pH treatments. Blank symbols and dotted line indicate bellowing detection limit.

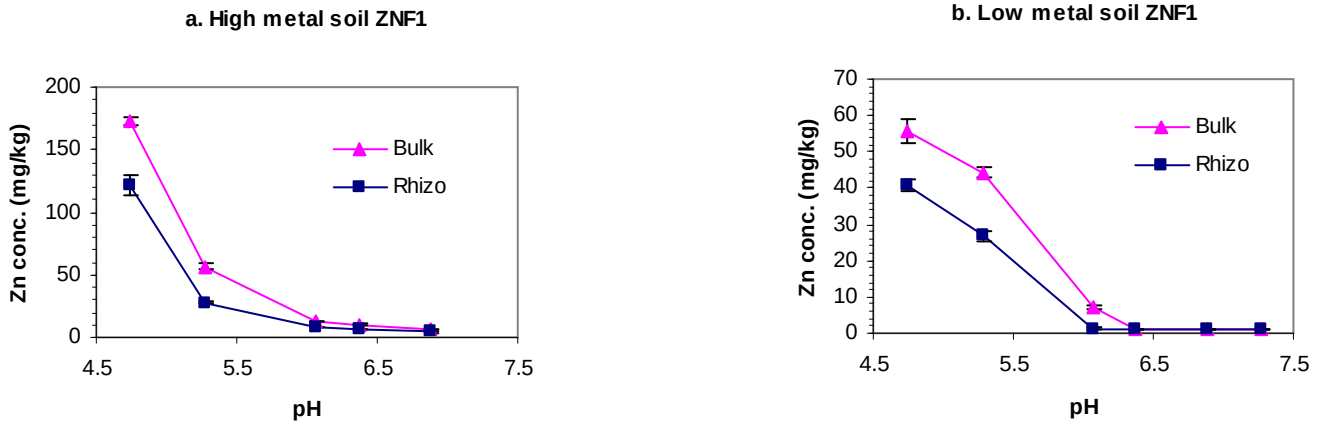

Figure 3.24. 0.1 M $Sr(NO_3)_2$ extractable Zn concentrations in high metal soil (a) and low metal soil (b) with different pH treatments.

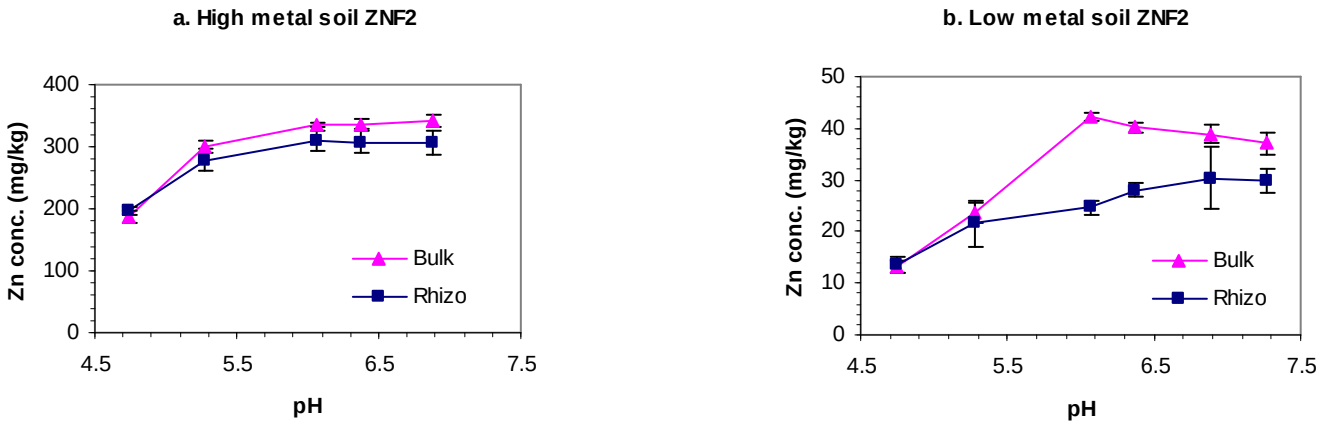

Figure 3.25. 1.0M NaOAc pH 5.0 extractable Zn concentrations in high metal soil (a) and low metal soil (b) with different pH treatments.

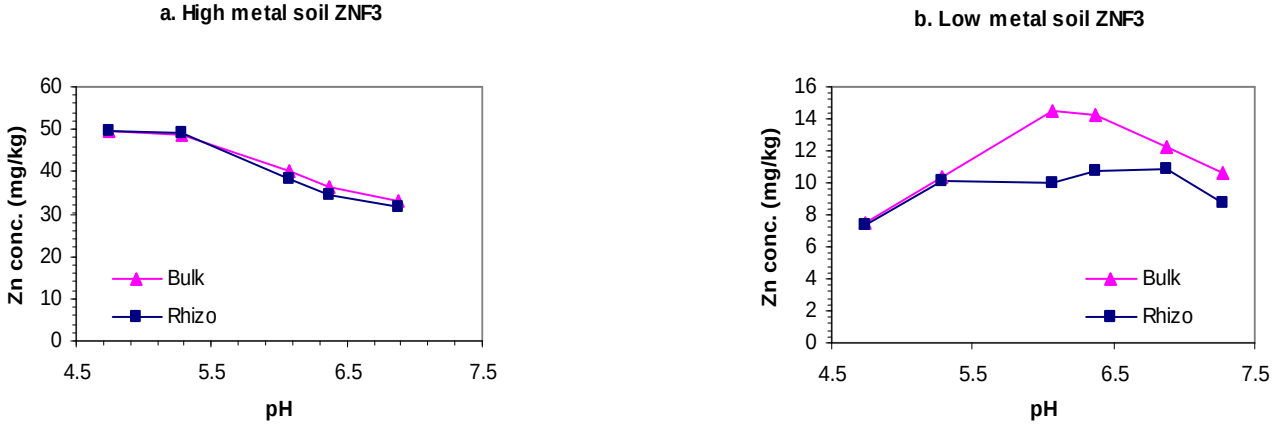

Figure 3.26. 5% NaOCl pH 8.5 extractable Zn concentrations in high metal soil (a) and low metal soil (b) with different pH treatments.

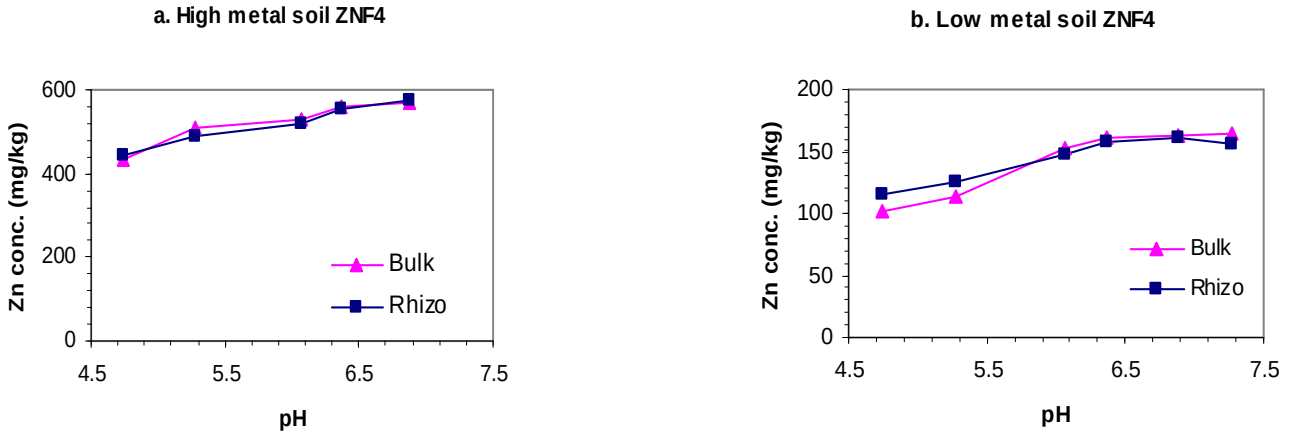

Figure 3.27. 0.4 M oxalate plus 0.1 M ascorbate pH 3.0 extractable Zn concentrations in high metal soil (a) and with metal soil (b) in different pH treatments.

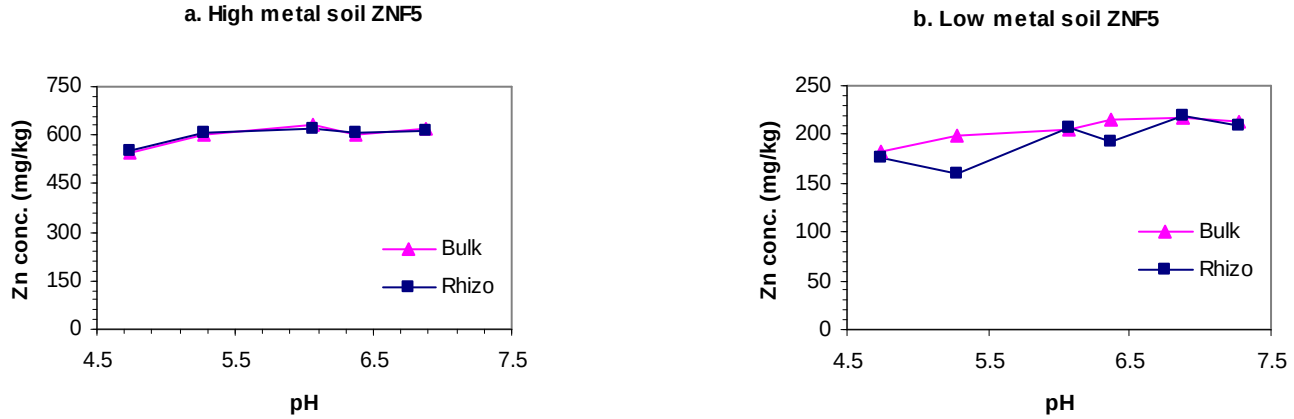

Figure 3.28. Residual Zn concentrations in high metal soil (a) and low metal soil (b) with different pH treatments.

Chapter 4: Changes in Soil Biological Activities under Reduced Soil pH during *Thlaspi caerulescens* Phytoextraction

ABSTRACT

Phytoextraction of soil Zn and Cd requires continual reduction in soil pH in order to maintain high metal uptake. Reducing pH of high metal soil, however, could negatively affect soil ecosystem function and health. The objectives of this study are to obtain the quantitative causal relationship between pH and soil biological activities in two Zn and Cd contaminated soils and to investigate the relationship between metals and soil biological activities under low pH. Soils were adjusted to 5 or 6 different pH levels by sulfur addition, followed by salt leaching. *Thlaspi caerulescens* was grown for 6 months, and both the rhizosphere and non-rhizosphere soil biological activities were tested after harvest. Reducing pH significantly lowered soil alkaline phosphatase activity, arylsulphatase activity, nitrification potential, and respiration. However, acid phosphatase activity was increased with decreasing pH. The relationship between soil biological activities and pH was well characterized by linear or quadratic regression models with R^2 values ranging from 0.57 – 0.99. In general, the three enzyme activities, nitrification potential, and the ratio of alkaline phosphatase to acid phosphatase activity were very sensitive indicators of soil pH status while soil respiration was not sensitive to pH change. The rhizosphere soil had higher biological activities than non-rhizosphere soil. The negative effects observed in the non-rhizosphere soil were alleviated by the rhizosphere influence. However, rhizosphere soil showed lower nitrification potential than non-rhizosphere soil, probably due to substrate limitation in our study.

Key words: soil pH, biological activity, rhizosphere, non-rhizosphere soil, *Thlaspi caerulescens*

4.1 INTRODUCTION

Phytoextraction uses unusual hyperaccumulator plants to accumulate high quantities of metals in plant biomass. It offers a low cost strategy to clean up contaminated soils and the plant ash may also have economic value (Baker et al., 1994; Chaney et al., 2000). The hyperaccumulation process involves rapid uptake, high rates of translocation from roots to shoots, and huge storage capacity by vacuolar compartmentalization (Chaney et al., 1997). However, the first step is uptake rate-limiting and thus critical to phytoextraction success. Plant uptake is generally limited by metal availability. Increasing metal availability usually results in enhanced uptake and higher shoot metal concentration (Brown et al., 1995 a).

The success of phytoextraction depends on appropriate soil management practices to make metals more available to plants. Among the diverse strategies to enhance phytoextraction, pH adjustment has received the most attention, because bioavailability of heavy metal is largely controlled by soil pH. Theoretically, lowering pH will increase metal availability. Studies conducted on other crops have shown a negative correlation between soil pH and metal transferred to plants (Narwal et al., 1983; Castilho and Chardon, 1995). Only a few studies have examined the soil pH effect on *T. caerulescens* hyperaccumulation (Brown et al, 1994; Brown et al., 1995 b).

Although reducing soil pH appears to be an effective strategy to enhance phytoextraction, precaution is needed because low pH and elevated metal concentrations may cause negative impacts to already vulnerable soil ecological systems. Do we increase phytoextraction without creating a further threat to the soil quality? This

question should be answered before any real world practice is allowed to take place. No such ecological risk assessment work, however, has been reported.

Although pH is a master variable, the causal relationship between pH and soil biological activity is rarely studied. Studies have been conducted to observe the correlations between diverse soil properties and soil biological activities, however, no conclusions about the effect of pH can be drawn since these studies were not controlled experiments to observe pH effects as an independent variable. Soil is a complex ecosystem; reactions in soil are different from those in a simplified chemical solution. The complexity of soil the micro-environment, the co-existence of copious numbers of microorganisms, the extensive interaction between different physicochemical reactions make it difficult to extrapolate the results of studies in a simplified system to the soil ecosystem.

Numerous studies have investigated liming effects on soil quality improvement (Arnold et al., 1994; Grego et al., 2000; Neale et al., 1997). Although soil pH is increased as a result of liming, the relationship between pH and soil quality indicators is not obvious. This is becasue liming causes many soil property changes in addition to pH. Change in soil pH is also a responsive variable, therefore no effect can be discussed with pH as an explanatory variable. Lastly, studies aimed at liming combined with other soil management practices, makes it even more difficult to examine the pH effect.

Furthermore, unlike healthy soil eco-systems, reducing soil pH in metal-rich soils may be complicated due to the increased bioavailable metal concentrations. To what extent this will contribute to the negative impacts on soil biological activities in addition to the low pH effect is unknown.

Soil quality is defined as the capacity of soil to fulfill its unique ecosystem functions. Nutrient recycling is one of the vital functions performed by soil. Scientists have been trying to develop soil quality indices for decades. Among them, acid phosphatase and alkaline phosphatase are important in the phosphorus cycle; they may provide insight in the soil organic phosphorus mineralization potential and microbiological activity of soils; arylsulphatase activity is important in S cycling; nitrification is the soil microbial process in which ammonium (NH_4^+) is transformed into nitrate (NO_3^-); Heterotrophic CO_2 respiration is a key process regulating carbon cycling in the biosphere. These five fundamental soil biological activities which play pivotal roles in the recycling of C, N, P, and S were selected to investigate the effect of pH in two Zn and Cd contaminated soils.

Our primary objectives in this study were (1) to obtain the quantitative causal relationship of pH and soil biological activity in soils which are acidified to increase Zn and Cd phytoextraction, (2) to compare the differences in the soil biological activities of non-rhizosphere and rhizosphere soil of *Thlaspi caerulescens* and how they differentially respond to reduced pH, (3) to investigate and compare the sensitivity of the different soil biological activities to pH change, and (4) to study how metal bioavailability is affected by pH change and how this in turn, further affects soil biological activities.

4.2 MATERIALS AND METHODS

4.2.1 Site description and soil sampling

Soil samples were collected from the A horizon of two cultivated fields in the proximity of a former Zn smelter that had been in operation for nearly 100 years at Palmerton, PA. Metals released to the environment were primarily Zn and Cd resulting in a metal concentration gradient according to distance and direction from the smelter. Two soils were collected. One was about 4.5 km up wind from the smelter and characterized by relatively lower metal concentrations. The second soil collected was about 1.4 km down wind from the smelter, and contained higher metal content (Table 3.1). Both soils belong to the Montevallo series (loamy-skeletal, mixed, subactive, thermic, shallow Typic Dystrudepts). Soils were first passed through a 1 cm sieve to remove stones and large plant residues then passed through a 4 mm sieve. Soils were then homogenized and stored in closed containers to avoid dehydration.

4.2.2 Soil characterization

Total Zn and Cd concentrations in soil were measured by extracting with concentrated hot nitric acid and the extraction analyzed using a flame atomic absorption spectrometry. Soil particle size distribution was determined by hydrometer method (University of Maryland, 1978.). Soil pH was measured in a soil water suspension (10 g soil to 20 ml deionized water) after 1 h shaking on a reciprocal shaker at 180 rpm. Organic matter content was determined by loss on ignition. Plant available Ca^{2+}, Mg^{2+}, and K^{+} were extracted with Mehlich (I) and determined with a Technicon Auto-Analyzer using a colorimeter for Mg and a flame photometer for K and Ca. Total N was

determined by the combustion method. Plant available P was extracted with Mehlich (I) and determined with a Technicon Auto-Analyzer.

4.2.3 Soil pH adjustment and salt leaching

Different amounts of elemental sulfur (S) were used to adjust soil pH to desired levels based on a preliminary acid incubation experiment. Soil pH was monitored periodically by taking 10 g of soil and measuring the soil water suspension pH. Soil was thoroughly mixed every day to ensure equal distribution of sulfur and aerate the soil to speed up the S oxidation process. Completion of acidification was assumed when the same pH was measured for 3 consecutive weeks. Next, 500 ml of deionized water was used to leach salt from each pot. After all water drained and there was no surface water on the top of the soil, 500ml of additionaldeionized water was used to repeat the process. This process was repeated a third time.

4.2.4 Plant growth

Thlaspi caerulescens used in this research is a southern France type, collected from Viviez, France. The species has a very high Cd hyperaccumulation potential (Chaney, personal communication). Seeds were germinated and seedlings were grown for 60 days, with watering everyday to maintain relatively constant moisture. The flats were put into a controlled-environment growth chamber, which was set at 16h/8h day/night cycle at $25^{o}C/22^{o}C$. Light intensity was above 400 µmol photon m^{-2} s^{-1} and relative humidity was 65%. Peters® 20-20-20 general purpose fertilizer was used as a liquid spray when needed. Seedlings were then transplanted into 15 cm (diameter) by 14 cm (height) plastic pots.

Each pot contained 1 kg soil and received three plants. All pots were put into growth chambers. Chamber settings were the same as for the seedlings growth. After transplanting, the use of fertilizers was limited to avoid disturbing soil microbial systems. After another 6 months of growth, plants were harvested.

4.2.5 Rhizosphere soil sampling

Rhizosphere soil is defined as that portion of soil adjacent to and influenced by plant roots (Metting, 1993). *Thlaspi caerulescens* has a very prolific root system. After 6 months of growth, all soil in the pot was filled with fine roots. Therefore in this experiment, all the soil in the pot with plants was treated as the rhizosphere soil. At harvest, the shoot was cut using stainless steel scissors. Root and soil were manually separated.

4.2.6 Treatment structure and experimental design

A completely randomized block design with treatment in factorial combination was used with the following factors: 1) metal concentration (low and high), 2) presence of plant (w/ and w/o plant, i.e., rhizosphere soil and non-rhizosphere), and 3) soil pH (6.88, 6.37, 6.07, 5.28, 4.74). For the low metal soil, soil pH was adjusted to 6 levels; an additional pH treatment of 7.27 was used. There were 4 replications for each of the treatments which were randomly put into one of the four growth chambers.

For each pH treatment within the high or low metal soil, there were two pots, one had plants; the other one contained only soil. Both were maintained similarly and were

incubated under the same condition. After harvest, soils in the pots which had plant were rhizosphere soils, and soils in the pots without plant were non-rhizosphere soils.

4.2.7 Soil biological activities

Soil enzyme activities were measured by the colorimetric determination of p-nitrophenol released referring to a calibration standard curve (Tabatabai 1994). For each soil sample, controls without adding substrate mixture were also performed, and the p-nitrophenol concentration was subtracted from the sample's value. Triplicate samples were conducted for each soil sample.

Soil nitrification potential was measured by the shaken soil-slurry method (Hart et al., 1994). Ten gram of soil was mixed with 80 ml of nitrification substrate solution mixture and shaked at a reciprocal shaker at 300 rpm. A portion of the soil slurry was sampled at 6, 12, 18, 24, 36, and 48 h, centrifuged, and filtered using Whatman # 40 filter paper. The NO_3^- in the solution was analyzed by a colorimetric method. The rate of NO_3^- production was then calculated by linear regression of these results.

Soil respiration was determined by closed jar incubation with NaOH traps and followed by acid titration method (Zibilske, 1994).

4.2.8 Statistical analysis

Statistical analysis was conducted using SAS version 8.2 (SAS Institute, 2001). The assumption of normality was tested by examining the plot of residuals and calculating the Shapiro-Wilk statistic. Homogeneity of variance was tested by examining plots of predicted values versus residual values. The Spearman Test was used to test the

correlation between the predicted value and absolute value of the residual. Logarithm transformation was used when needed. After checking that the data met the assumptions, the PROC MIXED procedure was used for univariate ANOVA to determine the main factor and interaction effect with block as a random factor, the pH treatment of 7.27 in the low metal soil was omitted when doing this analysis. When significant effects were detected, pair-wise treatment mean comparisons were made using Least Significance Difference (LSD) t-test on pH treatment means. Linear or quadratic regressions were calculated by the least-squares method. Differences between non-rhizosphere soil and rhizosphere soil treatment means were compared by a paired t-test. The association between two variables was estimated by the Pearson product-moment correlation coefficient. Unless otherwise indicated, the statistical significance level was set as $p \leq 0.05$.

4.3 RESULTS

4.3.1 Soil properties

The two soils collected had quite different levels of heavy metals (Table 3.2). The low metal soil had a total Zn and Cd concentration of 450 mg kg^{-1} and 5.0 mg kg^{-1}. For the high metal soil, it contained 1500 mg kg^{-1} and 25.4 mg kg^{-1}, respectively. Soil pH, organic matter content, and particle size distribution were similar for the two soils. The low metal soil has a much higher CEC than that of the high metal soil suggesting the two soils may have different mineralogy despite the similarity in their particle size distribution. Accordingly, the low metal soil had a higher buffering capacity, and more

elemental sulfur was needed to achieve the low pH than was required for the high metal soil.

4.3.2 Alkaline phosphatase activity

pH, location, and metal all had a significant effect on alkaline phosphatase activity. There was also significant metal by pH interaction (Table 4.1). Reducing soil pH significantly lowered alkaline phosphatase activity in both soils although the response was quite different. The correlation coefficient with pH was 0.77 ($p<0.0001$) (Table 4.5).

For the high metal soil, from pH 6.88 to pH 6.07, activity declined slowly and from pH 6.07 to 4.74, activity decreased rapidly (Fig. 4.1 d). There was no significant reduction between the pH treatments of 6.88, 6.37, and 6.07. However, after pH 6.07, each lower pH treatment had significantly lowered alkaline phophatase activity than the previous higher pH treatment. For the low metal soil, the alkaline phosphatase activity showed an “S” curve pattern (Fig. 4.1 c). From pH 7.27 to 6.37, both the non-rhizosphere and rhizosphere soils alkaline phosphatase activities were increased with decreasing pH. From pH 6.37 to 5.28, activities declined rapidly. However, from pH 5.28 to 4.74, alkaline phosphatase activity stabilized for non-rhizosphere soil and declined only slightly for rhizosphere soil. For both soils, the rhizosphere soil had higher alkaline phosphatase activities than non-rhizosphere soil. However, the differences were less at lower pH.

4.3.3 Acid phosphatase

Acid phosphatase activity was significantly affected by pH, location, metal, and location by pH, metal by pH interactions (Table 4.1). In contrast to alkaline phosphatase, acid phosphatase activities generally increased with decreasing soil pH. The correlation coefficient with pH was -0.79 ($p<0.0001$) (Table 4.5). For this enzyme, the low metal soil had higher activity than the high metal soil.

For the high metal non-rhizosphere soil, each lower pH treatment had significantly higher activity than the previous higher pH. For the rhizosphere soil, there was no significant difference between pH treatment of 6.88 and 6.37. After that, each lower pH treatment significantly increased the activity (Fig. 4.1 b).

For low metal soil, from pH 6.88-5.28, acid phosphatase activity was increased with decreasing pH, but declined at the lowest pH for both the non-rhizosphere and rhizospshere soils (Fig. 4.1 a). For the non-rhizosphere soil, the highest activity was at pH 5.28. For the rhizosphere soil, the highest activity was at pH 5.28 and 4.74. For both soils, at higher pH, rhizosphere soil had higher acid phosphatase activity than non rhizosphere soil, and this relationship was reversed at lower pH levels.

4.3.4 Arylsulphatase

Arylsulphatase activity was significantly influenced by pH, location, metal, and location by pH, metal by pH interactions (Table 4.1). Overall, arylsulphatase activities decreased with decreasing soil pH. The correlation coefficient with pH was 0.91 ($p<0.0001$) (Table 4.5).

For the high metal soil, the rhizosphere soil had higher arylsulphatase activity than the non-rhizosphere soil. But again, this difference tended to be smaller at the lowest pH level (Fig. 4.1 f). For non-rhizosphere soil, each lower pH treatment had significantly lower arylsulphatase activities than its previous higher pH treatment. This was also true for the rhizosphere soil except that there was no significant difference between pH treatment of 6.88 and 6.37.

For the low metal soil, the activity curve of rhizosphere soil was similarto the non - rhizosphere soil, with the former always being a little higher than the latter at each pH level (Fig. 4.1 e). For both non-rhizosphere and rhizosphere soils, there was no significant difference between pH treatments of 7.27, 6.88, and 6.37. However, after that, each lower pH treatment significantly reduced the activity compared to its previous higher pH treatment.

4.3.5 Nitrification potential

Only pH and the location by metal interaction had a significant effect on nitrification potential (Table 4.1). Nitrification potentialw as generally decreased with descending pH. The correlation coefficient with pH was 0.63 ($p<0.0001$) (Table 4.5).

For the high metal soil, the nitrification potential first increased with decreasing soil pH. The highest nitrification rate was observed at the second highest pH level. Then, it continually decreased with the decreasing of pH (Fig. 4.2 b). For both the non-rhizosphere and rhizosphere soils, there were no significant differences between the pH treatments of 6.37, 6.07, and 6.88. Then a significant reduction of nitrification occurred when pH was further reduced.

For the low metal soil, the nitrification potential declined with decreasing pH with only one exception – the lowest rate for non-rhizosphere soil was at pH 5.28 (Fig. 4.2 a). A significant reduction occurred when pH was below 6.37.and 6.07 for the non-rhizosphere and the rhizosphere soils, respectively. Surprisingly, the non-rhizosphere soil had much higher nitrification potential than the rhizosphere soil for both soils.

4.3.6 Soil basal respiration

pH, location, and metal all had a significant effect on soil respiration (Table 4.1). Interestingly, this is the only activity that pH was not the major influencing factor. The highest F value was for location, which was 109 while the F value for pH was only 3. The correlation coefficient with pH was 0.25 ($p<0.05$). For both soils, the rhizosphere soil had much higher basal respiration rate than the non-rhizosphere soil.

Apparently, pH acidification sometimes stimulated basal respiration. For example, for the high metal rhizosphere soil, the highest respiration rate appeared at pH 5.28. Even when soil pH reached low as 4.74, soil still had the same level of respiration as that of the highest pH. Overall, there was no statistically significant difference among the pH treatments for the rhizosphere soil. For the non-rhizosphere soil, there was no significant difference between the four higher pH treatments. But when pH was reduced to 4.74, respiration was significantly reduced (Fig 4.2 d).

For the low metal, non-rhizosphere and rhizosphere soils, respiration continually declined with decreasing soil pH (Fig. 4.2 c). From pH 7.27 to pH 6.88, respiration was significantly reduced. There was no significant difference between pH treatments of 6.88,

6.07, 6.37, and 5.28. But when pH was reduced to 4.74, respiration was significantly reduced again.

4.3.7 0.1 M $Sr(NO_3)_2$ extractable metal concentration

0.1 M $Sr(NO_3)_2$ extractable metal concentrations are seen as the bioavailable and exchangeable forms for heavy metals and is closely related to plant uptake and metal toxicity. Data showed that the concentration of this form of metal was strongly controlled by pH. Changing pH significantly affected all six observed metal concentrations. Decreasing pH drastically increased the concentration of Al, Cd, Mn and Zn. The concentration of Ca and Mg was reduced at lower pH. Notably, although Al concentration increased with decreasing of pH in both soils, the extent of increase was not the same for the high and low metal soils. For the high metal soil, from the highest to the lowest pH, Al increased about 30%. However, for low metal soil, there was an 8 to 11 fold increase. The final concentration in the low metal soil reached 49 and 71.8 mg kg^{-1} for rhizosphere soil and non-rhizosphere soil, respectively. This suggests that the two soils may have different mineralogy. This phenomenon is consistent with the higher buffering capacity of low metal soil previously observed when S was added to soil. The major differences between the high and low metal soils were Cd and Zn concentrations, with the former was much higher than the latter. However, there was not much difference in the concentrations of Al, Ca, and Mg between these two soils. Rhizosphere soil generally had lower 0.1 M $Sr(NO_3)_2$ extractable metal concentrations, especially of Cd, and Zn, indicating the uptake by plant roots lowered the available metal concentrations around the roots.

4.4 DISCUSSION

4.4.1 Sensitivity of different soil biological activities responding to pH change

There have been increasing interests in developing methodologies which are indicators of soil health and sustainability, reflecting changes in soil properties. The focus has switched from simple chemical approaches to more integrated biological approaches. Soil microbial-mediated processes viewed as an integration of soil physical, chemical, and biological characteristics therefore are excellent candidates to reflect changes in soil conditions. Our data demonstrate that soil biological activities were extremely sensitive to pH change. At the tested pH range-from pH 4.74 to 6.88 or 7.27 for high or low metal soil, respectively, pH was the most important factor influencing soil biological activities. Except for acid phosphatase, lowering pH significantly reduced all activities. The 50% of inhibition occurred at ΔpH -1.85, -1.42, -1.55, -3.0 for alkaline phosphatase, arylsulphatase, nitrification potential, and respiration, respectively. The degree of inhibition is strongly affected by change in pH (Fig. 4.3). Opposite to other tested activities, acid phosphatase activities increased with decreasing pH. So we calculated the inhibition of this enzyme based on the lowest pH levels when its activity is the highest, the 50% of inhibition was at ΔpH 2.22.

There are several proposed mechanisms that explain sensitivity of enzymes to pH changes. Ionization or deionization of the acidic or basic groups in the enzyme active center accounts for most of the decline in enzyme activity when pH deviates from optimum. Soil pH can change the concentration of inhibitors or activators, as well as the substrate in soil. pH stability of soil enzymes is also highly dependent on the soil properties (Frankenberger et al., 1982). Changes in enzyme activities may reflect the

changes in number and relative composition of soil microbes in relation to pH change. In a study of phosphatase and arylsulphatase activities in wetland soils (Kang and Freeman, 1999), pH was found to be positively correlated with phosphatase activity. Acosta-Martinez and Tabatabai (2000) also observed a significant and positive correlation between alkaline phosphatase and arylsulphatase activity with pH-the correlation coefficient were 0.89 and 0.66-respectively and a negative correlation of acid phosphatase and pH with a correlation coefficient -0.69. Stuczyuski et al. (2003) found that changes in pH after salt amendments may be responsible for some of the inhibition effects in soil biological activities previously being attributed to the metal toxicity. However, in these studies, the expainatory variables were not pH, therefore strong correlations do not necessarily imply a direct pH effect.

Bacteria involved in nitrification are presumably sensitive to pH. Our data illustrated the strong pH sensitivity of nitrification. The correlation coefficients of nitrification potential with pH was 0.63 ($p<0.0001$). Similarly, in a study of nitrification potential in Pb or Cu contaminated soils (Sauve et al., 1999), pH appeared to be the most influential parameter. Soil heterotrophic respiration involves numerous soil micro-organisms. Under stress, such as low pH, some sensitive organisms may die, while other tolerant organisms may survive. Some acid-loving organisms, such as acidophilus, may even flourish. As a sum of the various responses, it is not surprising to observe that soil respiration is not as sensitive to pH change as enzyme activities and nitrification potential. The plot of percentage of inhibition versus ΔpH has the lowest R^2 value, 0.43, among the five activities. Respiration also had the lowest value of correlation coefficient with pH, $r=0.25$ ($p<0.05$). The effect of pH on soil respiration was investigated by several other studies.

For example, in acidic aquatic ecosystms, marked inhibition of decomposition of organic matter has been observed (Traaen 1980, McKinley and Vestal 1982). Reduced soil respiration was also observed by Speir et al. (1999) due to metal addition and acidification. And soil respiration responded differently to acid amendment in each of their tested soils.

4.4.2 What can the ratio of alkaline phosphatase activity to acid phosphatase activity tell us?

Our data of the alkaline phospatase and acid phosphatase activities support other researchers' findings that alkaline phosphatase activity was predominant in neutral or alkaline soils, while acid phosphatase activity was predominant in acid soils (Eivazi and Tabatabai, 1977; Dick and Tabatabai, 1984). As pH decreased, the ratio of alkaline phosphatase activity (AlP) to acid phosphatase activity (AcP) decreased accordingly. At pH 4.74, the AlP/AcP ratio was also the lowest for all the four types of soils (high non-rhizosphere, high rhizosphere, low non-rhizosphere, low rhizosphere) with a very narrow range, from 0.12 to 0.14. At the highest pH, there was an approximately 9-fold increase in the AlP/AcP ratio, with a range from 1.03 to 1.15 in the four types of soils. There were very good linear regressions for AlP/AcP ratio with pH. The R^2 values were 0.90, 0.94, 0.97, and 0.98 for the four soil types (Fig. 4.4). This indicates that it is possible to assess AlP/AcP ratio based on soil pH, and vice versa. Previously, Dick and Tabatabai (1992) proposed the idea of using alkaline phosphatase and acid phosphatase activities to assess effective soil pH. This was further developed by Dick et al. (2000). They reached a conclusion that when a soil has an AlP/AcP ratio greater than 0.5, the soil pH should be

approximately 6.0. Our data were consistent with their observations in that the AlP/AcP ratio is, indeed, a sensitive indicator of soil pH status. However, in our study, only one type of soil, low metal non-rhizosphere soil, reached pH 6.05 when the ratio was 0.5. For the low metal rhizosphere soil, high metal non-rhizosphere soil, high metal rhizosphere soils, the pH were 5.84, 5.49, and 5.21, respectively. At these pH levels, soil biological activities may have already been negatively affected. So whether an AlP/AcP ratio of 0.5 can divide soils into two groups is questionable. This indicator might therefore be combined with other soil biological measurements to ensure a truly appropriate soil pH evaluation.

4.4.3 Difference in the soil biological activities between the non-rhizosphere soil and rhizosphere soil of *T. caerulescens*.

Rhizosphere soil has long been known to be different from non-rhizosphere soil. A number of rhizodeposition products (root exudates, cell lysates, mucilage, secretions, etc.) make rhizosphere soil a favorable environment for microbes to thrive. Bacteria of the rhizosphere are physiologically more active than non-rhizosphere soil bacteria. Accordingly, we observed higher biological activities in the rhizosphere soil than in the non-rhizosphere soil in general. Soil alkaline phosphatase, arylsulphatase, and soil respiration are consistently higher in the rhizosphere under all pH treatments. It is interesting to note that rhizosphere soil had lower nitrification potential than non-rhizosphere soil in most cases indicating lower number of nitrfiers in the rhizosphere. During the experimental period, we only applied a minimum amount of fertilizer in order to avoid disturbing the soil microbial populations. The rhizosphere soil was most likely

depleted of available nitrogen, i.e., NH_4^+, which is the substrate for nitrifiers. Therefore it is not so surprising that substrate limitation resulted in lower numbers of nitrifying bacteria in the rhizosphere soil.

Plant roots can also cause considerable changes in the rhizosphere pH (Hinsinger, 1998; Jaillard et al 2001). Depending on the forms of nitrogen used by the plant, this change could be acidification or alkalinization (Smiley et al., 1974; Römheld, 1986; Gahoonia et al., 1992). The contribution of organic acid exudation to rhizosphere acidification varied in different studies (Haynes, 1990; Jones and Darrah 1994; Jones et al., 1994; Hinsinger, 1998; Jones, 1998; Dindelaker et al., 1989; Ryan et al., 1995). Our data indicate a slight pH increase (about 0.05-0.3 units, data not shown) in the rhizosphere soil after harvest. Nevertheless, although *Thlaspi caerulescens* was found to be able to mobilizing nonlabile forms of metals (McGrath et al., 1997; Whiting et al, 2001 a), it appeared that it did not take advantage of acidification to achieve that. This has also been observed by several other studies (Knight et al., 1997 a; Luo et al., 2000). The slight increase in rhizosphere pH may explain part of the reason that rhizosphere soil had higher biological activities than non-rhizosphere soil in our study.

4.4.4 Correlations between soil metals and soil biological activities.

Numerous studies have documented the adverse effects of heavy metals on soil biological activities. However, there is also disagreement in the current literature. Our data demonstrated the strong correlations between soil biological activities and 0.1 M $Sr(NO_3)_2$ extractable metal concentrations. Based on the sign of the correlation coefficients, we can partition these 6 metals into two groups: one is Al, Cd, Mn and Zn;

the other includes Ca and Mg. The correlation coefficients between the first group and alkaline phosphatase, arylsulphatase, nitrification potential and respiration were all negative values. While the correlation coefficients between those activities and the second group were all positive. Acid phosphatase had an opposite relationship with extractable metals from the other four activities. All correlation coefficients were significant at the 0.05 level except arylsulphatase with Mg and nitrification potential with Mg. The order of the absolute values of coefficients between these metals and soil biological activities were: Mn>Ca>Zn>Cd>Al>Mg. Magnesium is the metal least associated with microbial activities while Mn, Ca and Zn are highly associated. It appeared that the three enzyme activities were more affected by metals than nitrification potential and respiration. This was in agreement with other observations. In a study of heavy metal effect on a contaminated grassland ecosystem, significant reductions in enzyme activities were observed and the degree of reduction was closely associated with the degree of heavy metal contamination. The enzymes they tested included acid and alkaline phosphatases and several other enzymes (Kuperman and Carreiro, 1997). Arysulfatase was also found to be sensitive to heavy metals. Its activity is inhibited by a number of elements, including Cd and Zn (Al-Khafaji and Tabatabai, 1979). However, results about heavy metal stress on soil respiration are inconsistent in different studies. Metal salts added to three New Zealand soils significantly decreased soil respiration with a similar pattern: an initial sharp decline and then followed by a relatively constant activity or even slight increase. In a study of both smelter and laboratory-contaminated soils, the soil respiration rates of the most polluted samples were 54-77% lower than those of the control samples and were negatively correlated with the contamination level

(Nordgren et al., 1988). However, in a 120 day incubation study using cadmium-contaminated sewage sludge, soil respiration first decreased, but at the end of the incubation period, microbial respiration in the amended soils was significantly higher than those of the controls (Moreno, et al., 1999). The explanation for the increase according to the author is that the microorganisms increased their metabolic activity to combat the metal stress. Fliebbach et al. (1994)also observed increased respiration with increasing amount of heavy metals. In addition to the heterogeneity of soil microorganisms, the observed variation and insensitivity of respiration may also be due to the difference in metal source (i.e., metal salts vs. sludge-borne metals), the time of measurement after addition, soil properties, etc.

4.5 CONCLUSIONS

Several conclusions can be drawn from this experiment:

1) Reducing pH had a significant negative impact on soil microbial activity. Soil alkaline phosphatase activity, arylsulphatase activity, nitrification potential, and respiration were significantly reduced after acidification of soil.
2) Acid phosphatase activity responded to acidification differently from all the other tested parameters- it was increased with decreasing pH.
3) The three enzyme activities, nitrification potential, and the ratio of alkaline phosphatase to acid phosphatase activity were sensitive indicators of soil pH status while soil respiration was not sensitive to pH change.

4) The rhizosphere soil has higher biological activities than in thenon- rhizosphere soil. The negative effects observed in the non-rhizosphere soil were alleviated by the rhizosphere influence except for nitrification.

Table 4.1. Summary of analysis of variance of the effect of pH, location, metal, and their interactions on soil biological activities

ANOVA Source of variation	df	AlP	Ac P	Arylsulphatase	Nitrification	Respiration
		---------- F value ----------				
pH	4	124***	140***	245***	21.4***	3*
Location (L)	1	49***	8**	44***	1	109***
Metal (M)	1	48***	206***	5*	0	11**
pH x L	4	< 1	5**	3*	< 1	< 1
pH x M	4	12***	17***	6***	1	1
L x M	1	< 1	< 1	< 1	7**	1
pH x L x M	4	< 1	2	< 1	2	1

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 4.2. Regression models of biological activities on pH in high metal soil

Activity	Location	Regression Equation	R square
AlP	Non-rhizosphere	$Y = -3.21\times10^{3} + 1.10\times10^{3}\times pH - 81.2\times pH^{2}$	0.95***
	Rhizosphere	$Y = -45.4\times10^{3} + 1.59\times10^{3}\times pH - 121\times pH^{2}$	0.97***
AcP	Non-rhizosphere	$Y = 6.77\times10^{3} - 1.85\times10^{3}\times pH + 134\times pH^{2}$	0.99***
	Rhizosphere	$Y = 5.29\times10^{3} - 1.41\times pH\times10^{3} + 105\times pH^{2}$	0.97***
Arylsulphatase	Non-rhizosphere	$Y = -523 + 101\times pH$	0.96***
	Rhizosphere	$Y = -564 + 121\times pH$	0.94***
Nitrification	Non-rhizosphere	$Y = -1.66 + 0.57\times pH - 0.045\times pH^{2}$	0.64**
	Rhizosphere	$Y = -0.20 + 0.045\times pH$	0.81***
Respiration	Non-rhizosphere	$Y = -3.22\times10^{-4} + 1.60\times10^{-4}\times pH$	0.57**
	Rhizosphere	$Y = 1.85\times10^{-3} + 1.91\times10^{-5}\times pH$	0.80**

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 4.3. Regression models of biological activities on pH in low metal soil

Activity	Location	Regression Equation	R square
AlP.	Non-rhizosphere	$Y = -610 + 151 \times pH$	0.82***
	Rhizosphere	$Y = -3.54 \times 10^3 + 1.13 \times 10^3 \times pH - 75.9 \times pH^2$	0.86***
AcP	Non-rhizosphere	$Y = -2.79 \times 10^3 + 1.56 \times 10^3 \times pH - 157 \times pH^2$	0.87***
	Rhizosphere	$Y = 2.49 \times 10^3 - 265 \times pH$	0.82***
Arylsulphatase	Non-rhizosphere	$Y = -501 + 108 \times pH$	0.92***
	Rhizosphere	$Y = -542 + 116 \times pH$	0.92***
Nitrification	Non-rhizosphere	$Y = -0.22 + 0.059 \times pH$	0.81***
	Rhizosphere	$Y = -0.18 + 0.036 \times pH$	0.74***
Respiration	Non-rhizosphere	$Y = -7.98 \times 10^{-4} + 2.26 \times 10^{-4} \times pH$	0.69***
	Rhizosphere	$Y = -8.23 \times 10^{-4} + 4.41 \times 10^{-4} \times pH$	0.77***

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 4.4. Means and standard errors of 0.1M $Sr(NO_3)_2$ extractable metal concentrations

Soil type	pH	Al	Ca	Cd	Mg	Mn	Zn
		----------mg kg^{-1}----------					
	4.74	9.1[†](2.9[‡])	1371(30)	5.8(0.06)	272(24.4)	52.2(1.7)	158.0(2.9)
High	5.28	7.7(2.6)	1783(31)	2.8(0.08)	347(6.1)	27.2(1.1)	51.0(2.2)
non-	6.07	7.2(2.4)	2049(41)	1.1(0.03)	417(11.4)	11.5(1.0)	11.1(0.4)
rhizo	6.37	7.6(2.7)	2115(35)	0.9(0.05)	431(9.9)	10.0(0.5)	8.9(0.4)
	6.88	6.8(2.3)	2221(45)	0.7(0.02)	404(7.1)	6.9(0.4)	5.7(0.2)
	4.74	8.9(2.8)	1492(18)	1.2(0.11)	273(18)	51.3(1.7)	111.4(7.4)
High	5.28	7.9(2.8)	1911(13)	0.3(0.01)	365(8.1)	22.3(0.6)	25.0(1.0)
	6.07	6.7(2.3)	2072(29)	0.4(0.01)	432(3.8)	8.4(0.2)	6.9(0.4)
rhizo.	6.37	7.7(2.7)	2111(34)	0.3(0.01)	429(6.9)	7.6(0.2)	5.6(0.2)
	6.88	7.3(2.6)	2211(41)	0.3(0)	421(7.1)	5.5(0.2)	3.7(0.3)
	4.74	71.8(3.7)	976(114)	1.8(0.13)	87(8.7)	66.3(7.8)	52.3(3.3)
Low	5.28	15.3(2.4)	1157(58)	1.4(0.05)	132(16.9)	41.6(3.4)	41.0(1.3)
non-	6.07	6.1(2.0)	1511(20)	0.5(0.01)	153(11.2)	17.0(0.8)	6.4(0.3)
rhizo	6.37	5.7(2.1)	1854(40)	0.3(0)	133(5.9)	6.9(0.4)	0.9(0.0)
	6.88	5.7(2.1)	2068(33)	0.3(0)	117(8.3)	3.6(0.2)	0.8(0.0)
	7.27	6.2(2.2)	2397(47)	0.3(0)	117(2.9)	1.5(0.1)	0.8(0.0)
	4.74	49.0(7.3)	1077(130)	1.1(0.07)	69(6.2)	36.6(0.9)	38.3(1.7)
Low	5.28	23.6(2.4)	1574(80)	0.4(0.02)	106(9.8)	36.4(0.5)	24.6(1.5)
	6.07	5.7(1.9)	1721(35)	0.3(0)	152(3.0)	12.9(0.9)	1.1(0.1)
rhizo.	6.37	5.5(1.9)	1959(38)	0.3(0)	124(5.6)	5.2(0.3)	0.9(0.1)
	6.88	5.8(2.2)	2397(234)	0.3(0)	121(10.9)	2.9(0.2)	0.8(0)
	7.27	6.0(2.2)	2313(40)	0.3(0)	115(2.1)	1.4(0.1)	0.8(0)

[†] Values are the average of 8 observations, including 4 block replications and 2 laboratory duplicates for each block.

[‡] Values in the parenthesis are the standard errors calculated on 8 observations.

Table 4.5. Pearson correlation coefficients between soil biological activities and metal concentrations.
N = 88 Prob > |r| under H0: Rho=0

Activity	Al	Ca	Cd	Mg	Mn	Zn	pH
AcP	0.42***	-0.83***	0.37***	-0.43***	0.72***	0.51***	-0.79***
AlP	-0.45***	0.76***	-0.51***	0.30**	-0.81***	-0.62***	0.77***
Aryl	-0.41***	0.75***	-0.58***	0.10	-0.86***	-0.70***	0.91***
Nitri	-0.42***	0.53***	-0.37***	0.10	-0.59***	-0.50***	0.63***
Resp.	-0.34**	0.39***	-0.40***	0.21*	-0.30**	-0.23*	0.25*

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

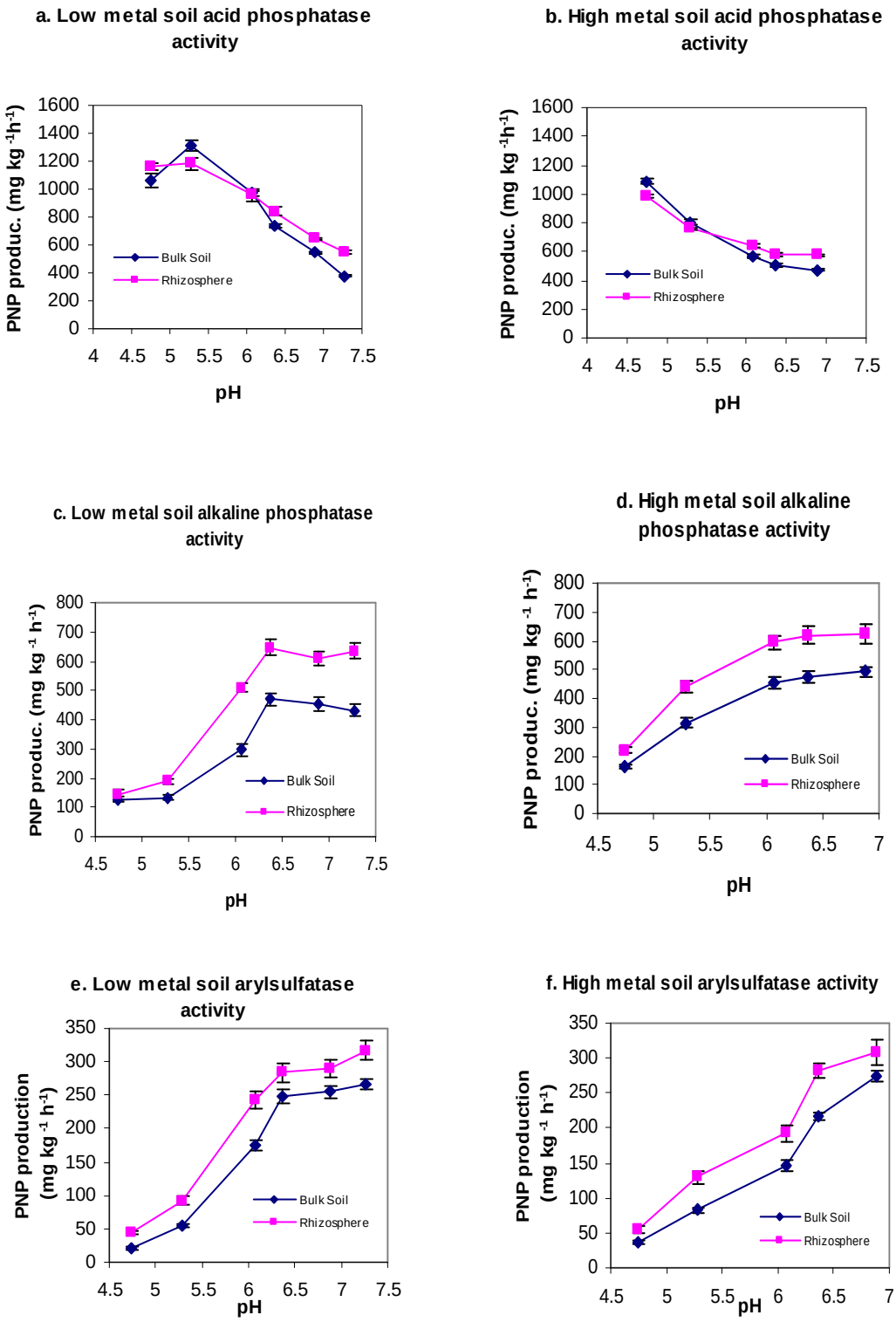

Figure 4.1. Means and standard errors of acid phosphatase, alkaline phosphatase, and arylsulphatase in different pH treatments. Each point is the average of 12 observations.

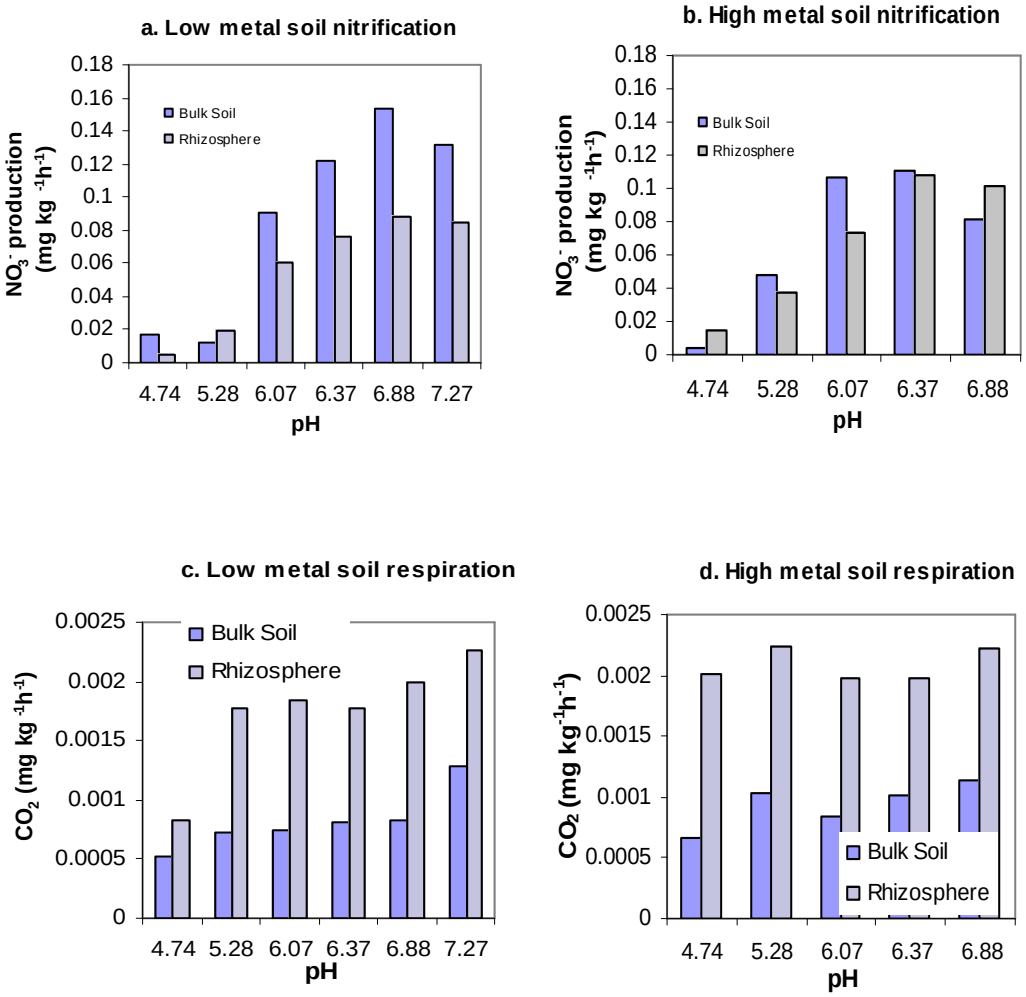

Figure 4.2. Means of nitrification potential and respiration in different pH treatments. For nitrification potential, each column is the average of 8 observations. For respiration, each column is the average of 4 replications.

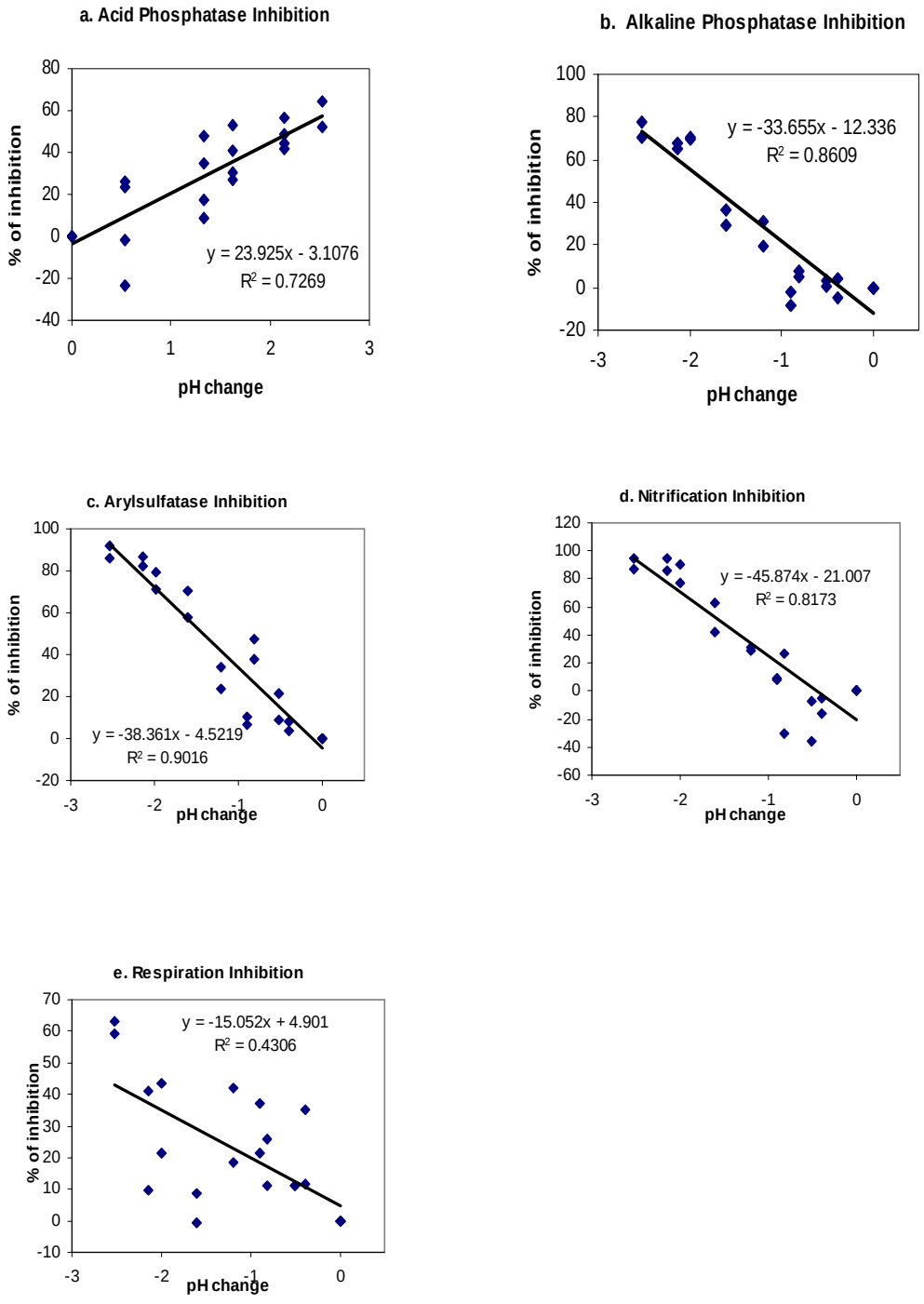

Figure 4.3 . Relationship between change in pH and inhibition in biological activities.

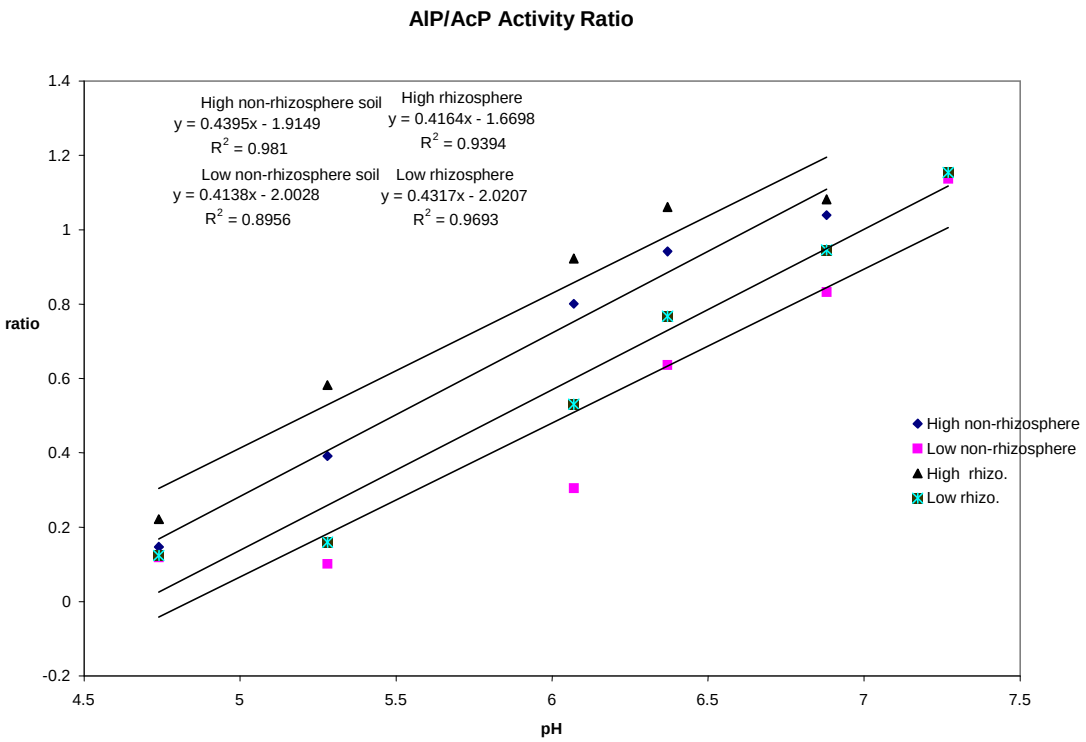

Figure 4.4. Relationship between the alkaline phosphatase to acid phosphatae activity ratio and pH.

Chapter 5: Changes in Soil Microbial Communities under Reduced pH during Cd and Zn Phytoextraction

ABSTRACT

Phytoextraction of soil Zn and Cd requires reduction in soil pH in order to maintain high metal uptake. Reducing pH of high metal soil, however, could negatively affect soil ecosystem function and health. Little is known about how soil microbial communities respond to the low pH stress at multiple community structure levels. In this paper, microbial population changes due to low pH during *Thlaspi caerulescens* phytoextraction from three perspectives of different hierarchies was studied. Soils were adjusted to 5 or 6 different pH levels by sulfur addition. *Thlaspi caerulescens* was grown for 6 months, and both the rhizosphere and non-rhizosphere soil microbial populations were tested after harvest. Reducing pH significantly shifted the community structure at different levels. Total soil microbial biomass was very sensitive to pH change. A reduction of only one unit of pH from the initial value reduced 50% of the total soil microbial biomass carbon. When pH was reduced by 2.2 units, soil microbial biomass nitrogen was reduced by 50%. Both the reduced biomass and the change in biomass C/N ratio suggest a change in community composition. This was further confirmed by plate counts of bacteria, actinomycetes, and fungi. Under low pH, the number of former two groups tended to decrease while the fungi tended to increase. As a representative of sensitive soil microorganisms, the population of indigenous *R. leguminosarum* bv. *trifolii* was reduced under low pH. At extreme pH, it could not be recovered from soil. We also found that the rhizosphere soil had higher microbial populations than the non-rhizosphere soil. But in most cases, the rhizosphere soil had no significant difference from the non-rhizosphere soil.

Key words: soil pH, microbial community, microbial populations, *Thlaspi caerulescens*

5.1 INTRODUCTION

Phytoextraction is proposed as a cost-effective and environmentally sustainable alternative to remediation of polluted soils. However, even metal uptake by hyperaccumulator plants is constrained by limited metal bioavailability. One major environmental factor that governs metal availability is pH. Theoretically, lowering pH will increase metal availability which in turn, will increase the metals transferred to plants. This has been confirmed by crop studies (Narwal et al., 1983; Castilho and Chardon, 1995). Only a few studies investigated the pH effect on *T. caerulescens* hyperaccumulation (Brown et al, 1994; Brown et al., 1995 b).

But pH, as a master variable in the soil environment, is a key factor in controlling soil ecological conditions. Reducing pH will affect many soil ecological characteristics. The ultimate goal of remediation of any kind is to regenerate a healthy soil ecosystem. If during the process, however, the soil health is further affected, it violates the remediation principal and phytoremediation will never be widely adopted. Therefore, prior to any real world remediation, it is important to determine to what extent the adverse impact would be and whether this affect is “acceptable”? However, no such ecological risk assessment work currently exists. Furthermore, reducing soil pH in metal-rich soils may have added ecotoxicity due to increased bioavailable metal concentrations. To what extent this will contribute to the negative impacts on soil microbial populations in addition to the low pH effect is unknown.

A healthy soil microbial community has two important components: species richness and species evenness. Maintenance of viable, diverse and functioning microbial communities is essential to soil quality since most of the major processes in the soil are carried out by soil microorganisms, such as C decomposition and nutrient cycling. However, it is extremely difficult, or virtually impossible to measure the entire range of microbial species in a given soil ecosystem. Therefore, choosing appropriate and sensitive indicator microorganisms which can reflect the soil ecosystem health becomes very important.

Soil total microbial biomass represents a small fraction, usually less than 5% of soil organic matter, but it plays a fundamental role in the cycling of all major plant nutrients, especially phosphorous, carbon, nitrogen, and sulfur (Nannipieri et al., 1990). Soil microbial biomass responds much more quickly than does total soil organic matter to changes in soil management (Powlson et al., 1987). It is also a sensitive indicator of changes in soil equilibrium (Kennedy and Papendick, 1995). Bacteria, actinomycetes, and fungi are three major groups of soil microbes. Legumes and other non-leguminous N_2-fixation symbionts have an important role in soil fertility and the global N-cycle.

Although efforts have been made to understand the effect of diverse environmental factors on soil microbial communities, little attention has been paid to the responses of soil microorganisms at multiple levels of community structure. In the present paper, through carefully selected representative variables, we examine microbial population changes due to low pH from three perspectives of different hierarchies. Specifically, the total soil microbial biomass C and N will reveal the changes in a macro sense; the

changes in the number of cultivable bacteria, actinomycetes, and fungi reflect the more specific influence of low pH as well as the degree of shifted community composition; and the investigation of the changes of the population size of rhizobium is an example of the consequence of stress on the specific sensitive species.

The primary objectives of this study were (1) to obtain the quantitative causal relationship of pH and soil representative microbial populations and to investigate changes of soil microbial community due to low pH through three different levels, (2) to compare the differences in the soil microbial populations of non-rhizosphere soil and rhizosphere soil of *Thlaspi caerulescens* and how they respond to reduced pH differently, (3) to investigate and compare the sensitivity of the different soil microbial populations to pH change, and (4) to study how metal bioavailability is affected by pH change and how this in turn, further affects soil microbial populations.

5.2 MATERIALS AND METHODS

5.2.1 Site description and soil sampling

Soil samples were collected from the A horizon of two cultivated fields in the proximity of a former Zn smelter that had been in operation for nearly 100 years at Palmerton, PA. Metals released to the environment were primarily Zn and Cd resulting in a metal concentration gradient according to the distance and direction from the smelter. We sampled two soils, one was at the west about 4.5 km up wind from the smelter and being characterized by relatively low metal concentrations; The other one was at the northeast about 1.4 km down wind from the smelter, containing higher metals (Table 3.2).

Both soils belong to Montevallo series (loamy-skeletal, mixed, subactive, thermic, shallow Typic Dystrudepts). Soils were first passed through a 1 cm sieve to remove stones and large plant residues then passed through a 4 mm sieve. Soils were then homogenized and stored in closed containers to avoid dehydration.

5.2.2 Soil characterization

Total Zn and Cd concentrations in soil were measured by extracting with concentrated hot nitric acid and measured by flame atomic absorption spectrometry. Soil particle size distribution was determined by the hydrometer method (University of Maryland, 1978.). Soil pH was measured in a soil water suspension (10 g soil to 20 ml deionized water). Organic matter content was determined by loss on ignition. Plant available Ca^{2+}, Mg^{2+}, and K^{+} were extracted with Mehlich (I) and determined with a Technicon Auto-Analyzer using a colorimeter for Mg and a flame photometer for K and Ca. Total N was determined by the combustion method. Plant available P was extracted with Mehlich (I) and determined with a Technicon Auto-Analyzer.

5.2.3 Soil pH adjustment and salt leaching

Different amounts of elemental sulfur (S) were used to adjust soil pH to desired levels based on a preliminary acid incubation experiment. Soil pH was monitored periodically by taking 10 g of soil and measuring the soil water suspension pH. Soil was thoroughly mixed every day to ensure equal distribution of sulfur and aerate the soil to speed up the S oxidation process. Completion of acidification was assumed when the

same pH was measured in three consecutive weeks. Then 500 ml of deionized water was used to leach salts from each pot. After all water drained and there was no surface water on the top of the soil, a second 500 ml of deionized water was used to repeat the process. This process was repeated a third time.

5.2.4 Plant growth

Thlaspi caerulescens used in this research is a southern France type, collected from Viviez, France with very high Cd hyperaccumulation potential (Chaney, personal communication). Seeds were germinated and seedlings were grown for 60 days, with watering everyday to maintain relatively constant moisture. The flats were put into a controlled-environment growth chamber, which was set at 16h/8h day/night cycle at $25^{o}C/22^{o}C$. Light intensity was above 400 µmol photon m^{-2} s^{-1} and relative humidity was 65%. Peters™ 20-20-20 general purpose fertilizer was used as liquid spray when needed. Seedlings were then transplanted into 15 cm (diameter) by 14 cm (height) plastic pots. Each pot contained 1 kg soil and received three plants. All pots were put into growth chambers. Chamber settings were the same for the seedlings growth. After transplanting, the use of fertilizers was limited to avoid disturbing soil microbial systems. After another 6 months of growth, plants were harvested.

5.2.5 Rhizosphere soil sampling

Rhizosphere soil is defined as that portion of soil adjacent to and influenced by plant roots (Metting, 1993). *Thlaspi caerulescens* has a very prolific root system. After 6

months of growth, all the soil in the pot was filled with fine roots. Therefore in this experiment, all the soil in the pot with plant growth was treated as the rhizosphere soil. At harvest, the shoot was cut using stainless steel scissors. Then the whole soil/root mass was taken out of the pot. Root and soil were manually separated.

5.2.6 Treatment structure and experimental design

A completely randomized block design with treatment in factorial combination was used with the following factors: 1) metal concentration (low and high), 2) presence of plant (w/ and w/o plant, i.e., rhizosphere soil and non-rhizosphere), and 3) soil pH (6.88, 6.37, 6.07, 5.28, 4.74). For the low metal soil, soil pH was adjusted to 6 levels; an additional pH treatment of 7.27 was used. There were 4 replications for each of the treatments which were randomly put into one of the four growth chambers.

5.2.7 Microbial biomass carbon (MBC)

Soil microbial biomass C was determined by the chloroform-fumigation-incubation method as described by Horwath and Paul (1994). Ten gram of moist soil was fumigated with vigorously boiled 50 ml ethanol-free chloroform for 30 s in a vacuum desiccator. This was repeated three times. The fourth time the chloroform was boiled for 2 min. Then the valve was closed and the soil together with the beaker of chloroform were incubated in the desiccator for 24 h. After fumigation, chloroform was removed and the desiccator was evacuated 3 min and flushed with air, this was repeated for eight times. Following removal of chloroform, both of the fumigated (F) and unfumigated (UF) soil samples

were put into glass mason jars containing 2 ml water to prevent soil desiccation. Then the air tight closed jars were incubated in the dark under room temperature for 10 d. After incubation, 2 ml of air was taken from the mason jar and injected into a 20 ml helium flushed vial. The amount of CO_2 was measured by gas chromatography. The amount of CO_2 in the vial was calculated by reference to a calibration curve plotted from results obtained with standard known concentrations of CO_2. The soil microbial biomass C was calculated by subtracting the amount of CO_2 in the control samples from the fumigated samples and adjusted by a correction factor as shown in the following equation:

$$\text{Biomass C} = (Fc - UFc)/Kc$$

Where Fc is the CO_2 flush from the fumigated sample,

UFc is the CO_2 flush from the unfumigated sample,

Kc is the fraction of biomass C mineralized to CO_2. It was taken as a common value of 0.41 in this experiment based on Anderson and Domsch (1978).

5.2.8 Microbial biomass nitrogen (MBN)

Following 10-d incubation, soils in the jar was transferred to 250 ml plastic cups and to which 50 ml 2 M KCl solution was added. The cups were capped, and shaken on a reciprocal shaker at 180 rpm for 30 min. After shaking, the soil suspension was filtered through Whatman #40 filter paper. The filtrate was analyzed for ammonium (NH_4^+) and nitrate (NO_3^-). MBN was calculated similarly to MBC as shown in the following equation:

$$\text{Biomass N} = (Fn - UFn)/Kn$$

Where Fn is the flush of NH_4^+ due to fumigation,

UFn is the NH_4^+ mineralized during 10 d incubation from a control, it is calculated as the amount of NO_3^- from a fumigated sample deduct the NO_3^- from an unfumigated control.

Kn is the proportion of microbial N mineralized to NH_4^+, it is taken as 0.54 in this experiment based on Jenkinson (1988).

5.2.9 Plate counting of bacteria, actinomycetes, and fungi

Enumeration of viable bacteria, actinomycetes, and fungi in soil sample was performed by the spread plate technique with different culture media. Ten gram of moist soil was added to 95 ml of sterile deionized water and mixed for 1 min at 22000 rpm in a Waring blender (Waring, New Hartford, CT). After allowing for settling for 1 min, 10-fold dilutions were made using sterile deionized water. Then 0.1 ml of aliquots from each of the appropriate dilutions were transferred to the agar plates. Three replicate plates were inoculated at each given dilution. The inoculated plates were then inverted and incubated in the dark in an incubator with temperature set at 28^{o}C. The number of bacteria, actinomycetes, and fungi were counted after 4, 4, 5 days of incubation, respectively. The incubation time was determined by a preliminary experiment.

The media used for culturing of viable bacteria were R2A (Reasoner and Geldreich, 1985) and RIM. Starch casein medium (Küster and Williams, 1964; Wellington and Toth, 1994) and Martin's medium (Wollum, 1982; Parkinson, 1994) were used or culturing of actinomycetes and fungi, respectively.

5.2.10 Estimation of population size of the indigenous *R. leguminosarum* bv. *trifolii*

The numbers of rhizobia able to nodulate white clover were determined by the most probable number (MPN) method (Weaver and Graham, 1994) using a 10-fold soil dilution series. *Trifolium repens* (white clover cv. Menna) was used as the trap host plants for *R. leguminosarum* bv. *trifolii*. Five replicate plant infection tubes were inoculated with 1 ml aliquots at each dilution step and the tubes were placed in a controlled environment growth chamber. The chamber was set at 12h/12h day/night cycles. Temperature was maintained at 28^{o}C during the day period and 24^{o}C during the night. Light intensity was above 400 µmol photon m^{-2} s^{-1}; relative humidity was 65%. Five replicate tubes inoculated with *R. leguminosarum* bv. *trifolii* USDA 2055 obtained from the USDA Rhizobium Germplasm Collection at Beltsville, MD were performed as positive controls for each set of samples, as well as five replicate tubes inoculated with sterile deionized water as negative controls. A check of nodulation was performed after 3 wk. Numbers of rhizobia were calculated using the MPNES computer program (Woomer et al., 1990; Woomer 1994).

5.2.11 Statistical Analysis

Statistical analysis was conducted using SAS version 8.2 (SAS Institute, 2001). The assumption of normality was tested by examining the plot of residuals and calculating the Shapiro-Wilk statistic; The homogeneity of variance was tested by examining a plot of predicted values versus residual values. The Spearman Test was used to test the correlation between the predicted value and absolute value of the residue. Logarithm

transformation was used when needed. After checking that data met the assumptions, the PROC MIXED procedure was used for univariate ANOVA to determine the main factor and interaction effect with block as a random factor, the pH treatment of 7.27 in the low metal soil was omitted when doing this analysis. When significant effects were detected, pair-wise treatment mean comparisons were made using the Least Significance Difference (LSD) t-test on pH treatment means. Linear or quadratic regressions were calculated by the least-squares method. Differences between non-rhizosphere soil and rhizosphere soil treatment means were compared by the paired t-test. The association between the two variables was estimated by the Pearson product-moment correlation coefficient. Unless otherwise indicated, all statistical significance levels were set at $p \leq 0.05$.

5.3 RESULTS

5.3.1 Soil viable bacteria using R2A medium

The number of colony forming units (CFU) of bacteria using R2A medium was significantly affected by pH, location, and soil metal (Table 5.1). It tended to decrease with the reduction of pH (Figure 5.1 a, b). The correlation coefficient with pH was 0.36 ($p<0.01$) (Table 5.3).

The highest numbers of bacteria were found at the higher pH treatments. But there were no significant differences between all five pH treatments both of the non-rhizosphere soil and rhizosphere soil for the high metal soil. For the low metal non-rhizosphere soil, bacteria numbers were significantly reduced when pH was reduced to

5.28, while for rhizosphere soil, the significant reduction occurred at pH 4.74. For both high and low metal soils, rhizosphere soil had higher number of bacteria than the non-rhizosphere soil; however, this difference was not significant.

5.3.2 Soil viable bacteria using RIM medium

The results for bacterial enumeration obtained using RIM medium were similar to that of R2A medium. pH, location and metal allhad significant effects (Table 5.1). The correlation with pH was 0.30 ($p<0.01$), i.e., the number of bacteria tended to decrease with the reduction of soil pH (Figure 5.1 c, d).

For the high metal non-rhizosphere soil, there were no significant differences between all pH treatments. For the high metal rhizosphere soil, a significant reduction occurred at the lowest pH treatment. For the low metal non-rhizosphere soil, there was no significant difference between the three highest pH treatments. But at pH 6.07, bacteria numbers were significantly reduced. At pH 5.28 and 4.74, a further significant reduction was observed. For the low metal rhizosphere soil, the lowest pH treatment had significantly fewer bacteria than the other four pH treatments. The magnitude of the number of culturable bacteria using either RIM or R2A media gave similar results. For both high and low metal soils, it was around 10^7 g^{-1} of soil.

5.3.3 Soil viable actinomycetes using Starch Casein medium

The number of viable actinomycetes in soils was significantly affected by pH, location, and metal (Table 5.1). The rhizosphere had higher numbers of actinomycetes.

The correlation with pH was 0.31 ($p<0.01$); recoverable actinomycetes tended to decrease with the reduction of soil pH (Fig. 5.2 a, b).

For the high metal non-rhizosphere soil, the three higher pH treatments had significantly higher numbers of actinomycetes than the two lower pH treatments. For the rhizosphere soil, however, there was no significant difference between all five pH treatments. Generally, rhizosphere soil had higher numbers of actinomycetes than non-rhizosphere soil at all pH treatments, but the difference was not statistically significant.

5.3.4 Soil viable fungi using Martin's medium

The number of CFU of fungi was about 2 logs lower than that of bacteria and actinomycetes. It was significantly influenced by pH, location, metal and the location by metal interaction (Table 5.1). Different from the other two groups, the number of fungi tended to increase with reduced pH (Figure 5.3 a, b). The correlation coefficient with pH was negative ($r = –0.23$, $p < 0.05$). The highest numbers generally occurred at the lowest pH treatments.

For both high metal non-rhizosphere and rhizosphere soils, the highest number of fungi was at pH 4.74 while the lowest number was at pH 5.28 and 6.07, respectively. There was no significant difference between all pH treatments in the rhizosphere soil. For the low metal rhizosphere soil, the lowest pH treatment had a significantly higher number of fungi than the other pH treatments. And the highest pH treatment had significantly lower number of fungi than all other five pH treatments. There was no significant difference among the four intermediate pH treatments. In the non-rhizosphere soil, the pH

5.28 treatment had significantly higher number of fungi than at pH 7.27. There was no significant difference among the other pH treatments. Again, rhizosphere soil had more fungi than non-rhizosphere soil, but this difference was only significant at the low metal soil pH 4.74 and 6.88.

5.3.5 Soil microbial biomass N

Soil microbial biomass N was only significantly influenced by pH while location and metal had no effect (Table 5.1). For the high metal soil, microbial biomass N consistently declined with the reduction of pH (Figure 5.5 a). The pH treatments of 6.88 and 6.37 had significantly higher biomass N than the pH of 6.07. When pH was reduced to 4.74, a further significant reduction occurred. For the low metal non-rhizosphere soil, the two highest pH treatments had significantly higher biomass N than the two lowest pH treatments. For the rhizosphere soil, a significant reduction was seen at pH 5.28, but there was no significant difference between the other pH treatments (Figure 5.5 b).

5.3.6 Soil microbial biomass C

Soil microbial biomass C was significantly influenced by both pH and location (Table 5.1). It was most highly correlated with pH ($r= 0.86$, $p < 0.001$). For the high metal non-rhizosphere soil, microbial biomass C increased at the second highest pH treatment, and then linearly declined with decreasing pH. The biomass C in the three higher pH treatments was significantly higher than in the two lower pH treatments. The pattern of rhizosphere soil closely followed the non-rhizosphere with the former always being

slightly higher than the latter (Figure 5.4 a). For the low metal rhizosphere soil, there was no significant decline for the first four pH treatments. A significant reduction of microbial biomass C, however, occurred at pH 5.28 and pH 4.74 (Figure 5.4 b). The curve presents a signmoidal form, which fits a quadratic pH regression with R^2 of 0.85 ($p<0.001$). For non-rhizosphere soil, microbial biomass C first increased at the second pH treatment, then declined as pH was further reduced.

5.3.7 The most probable number of rhizobia

The number of white clover rhizobia in the soil was influenced by pH, location, metal, as well as all interactions (Table 5.1). With the reduction of pH, rhizobia were drastically reduced, even totally eliminated (Figure 5.6 a, b). For the high metal non-rhizosphere soil, a significant reduction occurred at pH 6.07, when pH was further reduced to 5.28 and 4.74, a second significant reduction in rhizobia number occurred. For rhizosphere soil, the four higher pH treatments had significantly higher number of rhizobia than the lowest pH treatment. For the low metal soil, from pH 7.27-6.07, rhizobia numbers remained relatively constant. However, after pH 6.07, numbers rapidly decreased with decreasing pH and reached zero at the lowest pH treatment.

5.4 DISCUSSION

5.4.1 Sensitivity of different soil microbial populations to pH reduction in Zn and Cd contaminated soils

Results showed that pH was the most important variable controlling soil microbial communities. Soil microbial populations using selective media were significantly affected by reducing pH at different levels. In general, lowering pH reduced viable microbial populations. With the highest pH as the initial starting point, 50 per cent inhibition of the population occurred at ΔpH -2.73, -2.08, and -2.54 for bacteria using RIM medium, bacteria using R2A medium, and actinomycetes respectively. Interestingly, there were negative inhibition values, i.e., stimulating effect when pH was only reduced about 0.5 units. Unlike other groups, the population size of fungi increased with decreasing pH. So we calculated the inhibition of this population based on the lowest pH levels (pH 4.74) when its population was the highest or nearly highest, 50 per cent of inhibition occurred at ΔpH +3.18. It is easy to imagine that under acid environments, due to lack of competition from bacteria and actinomycetes, fungi may become the dominant microbial group.

Most of the soil microbial biomass C values were in the range of 100-200 $\mu g\, g^{-1}$ soil. In the literature, depending on the soil properties and degree of contamination, the MBC values had a wide range using chloroform-fumigation-incubation method (Bragato et al., 1998; Mendes et al., 1999; McCarty et al., 1998). Typically, most reported MBC values were between 100-300 $\mu g\, g^{-1}$ soil. MBC was extremely sensitive to change in soil pH. It was reduced by 50% by only one unit reduction in pH. This decrease in total soil

microbial biomass suggests changes in community structure since it is likely that not all species respond to stress evenly. Interestingly, at the two lowest pH values, the microbial biomass C had negative values. There may have several reasons for this. First, soil microbial biomass and microbial activities are two different concepts. Although in a healthy soil, high microbial biomass is generally correlated with high activities, in a stressed soil, this relationship is disrupted. Data for microbial number demonstrated that under long-term stress, there is a change in the microbial community. Disturbances favor communities dominated by small-bodied, rapidly reproducing hardy species (Woodwell 1983), while other sensitive species may die. The biomass of the soil ecosystem may hence decrease, but the activity (respiration) from the fewer resistant species may be still high, because "the repairing mechanism caused by the disturbance requires diverting energy from growth and production to maintenance. Hence, the R/B ratio (the maintenance to biomass ratio) increases" (Odum, 1985). The low biomass in a soil can not supply enough C to release CO_2 after fumigation to overcome the high respiration rate in the control soil. Also, a common observation in "stressed" soils is that the mineralization of native soil organic C is lowered. Thus, the soil is left with enhanced accumulation of soil organic matter. Therefore, providing extra lysed microorganisms through fumigation is not an effective way to stimulate respiration. These factors, plus some experimental error, make it possible for one to observe negative biomass values. Still, a soil ecosystem under long-term stress may become a "senile" ecosystem, characterized by low growth, low reproduction rate, high maintenance consumption. After fumigation, it is still not possible for the remaining living organisms to possess high

reproduction ability in a short period. Therefore, the surviving organisms cannot reach a high enough population during incubation so that the control soil had a higher amount of respiration than the fumigated soil. These three possible reasons may work alone or in combination to produce a negative biomass value. These negatives biomass values can be a further proof of the "worn out" condition of the soil microbial ecosystem under extreme stress.

In our soils, most soil microbial biomass N values were between 30-60 $\mu g\, g^{-1}$ soil. Soil microbial biomass N responded to pH changes similarly to biomass C, but to a less extent. The 50% reduction occurred when pH was reduced by 2.2 unit from its initial value. The slope of the linear regression curve of change in pH and inhibition in biomass N was much smaller than that of biomass C. Compared with biomass C, there seemed to be a 'lag time' in biomass N responding to pH changes. Disregarding the negative values, the microbial C/N ratio varied from 1-3 where pH did not seem to cause a consistent response. The reasons that caused the variation in the C/N ratios are unknown. We can only hypothesize that the composition of the microbial community and the nutritional status of the microorganisms must have undergone some type of shift or reconstruction. Theoretically, it is estimated that bacteria have a carbon to nitrogen ratio of 4/1 to 6/1 while fungal ratios generally fall between 10/1 to12/1. However, it is not uncommon for experimental methods to obtain much lower values. For example, McCarty et al (1998) reported typical values in their soil were around 2-4.

White clover rhizobia were very sensitive to change in pH. When pH was reduced by 1.6 unit, rhizobia were reduced by 50%. At the extreme low pH, rhizobia were no longer

found in the soil. The sensitivity of rhizobia to pH was also observed by Ibekew et al. (1997). They reported the soil pH rather than metal concentration caused difference in genetic structure and phenotypic characteristics of clover rhizobia isolated from metal contaminated soils. Effective isolates were associated with higher pH levels while ineffective isolates were associated with lower pH levels. In this experiment, our purpose was only to examine the existence of rhizobia by nodulation and did not discriminate between nodulation and effective nodulation.

5.4.2 Correlations between metal and soil microbial populations

Correlation coefficients between the population size of bacteria and actinomycetes with 0.1 M $Sr(NO_3)_2$ extracble Al, Cd, Mn, Zn were all negative while with Ca and Mg values were all positive. Many correlations were significant, but most of the absolute coefficients values were below 0.4. Fungi were significantly and negatively correlated with Ca and Mg, as was soil microbial biomass N. The low association between metal and the population of three major groups indicate that the metal effect was not a dominant factor governing microbial communities in this experiment. However, studies suggest that heavy metal stress may change the soil microbial community structure and lead to a decrease in the microbial diversity. Our data indicated that soil microbial biomass C and the rhizobia were the variables that were most influenced by metal concentrations at low pH. Both were significantly negatively correlated with Al, Cd, Mn, and Zn, with correlation coefficients mostly above 0.6. The only measured metal that was not correlated with either was Mg. These findings were consistent with the literature where

there were documented sensitivity of soil microbial biomass C and rhizobium to heavy metal stress. Studies reported a decrease on soil microbial biomass as a result of heavy metal stress (Chander and Brooks, 1991, 1993; Ellis et al., 2001; Frostegård et al., 1993, 1996; Kelly et al., 1999; Knight et al., 1997 b; Leita et al., 1995). There are also a number of studies related to heavy metal impact on N_2-fixation especially on leguminous symbiotic N_2-fixation. The observed influences include decreased population size of these organisms (Chaudri et al., 1992, 1993, 2000 a, 2000 b; Giller et al., 1993), decreased genetic diversity (Giller et al., 1989; Hirsch et al., 1993), delayed nodulation (El-Kenawy et al., 1997), and ineffective nodulation (Chaudri et al., 1992; McGrath 1995).

5.5 CONCLUSIONS

Reducing pH significantly shifted the community structure. This conclusion was based on three different levels of observations.

1) Both the reduced biomass and the change in biomass C/N ratio suggested a change in community composition.
2) This was further confirmed by plate counts of bacteria, actinomycetes, and fungi. Under low pH, the number of former two groups tended to decrease while the fungi tended to increase.
3) Third, as a representative of soil sensitive microorganisms, the population of indigenous *R. leguminosarum* bv. *trifolii* was greatly reduced under low pH.

We also found that rhizosphere soil had higher microbial populations than non-rhizosphere soil.

Table 5.1. Summary of analysis of variance for the effect of pH, location, metal and their interactions on soil microbial populations

ANOVA Source of variatin	df	Bacteria (rim)	Bacteria (r2a)	Actino-mycetes	Fungi	Biomass C	Biomass N	MPN
		---------- F value ----------						
pH	4	4.7**	3.9**	4.0**	2.9*	79.2***	18.2***	135***
Location (L)	1	14.0***	13.9***	24.6***	31.4***	9.9**	0.2	77.0***
Metal (M)	1	13.5***	6.8*	4.6*	5.0*	1.5	3.0	25.1***
pH x L	4	0.6	0.4	0.1	1.0	0.5	0.4	3.9**
pH x M	4	1.0	0.6	0.1	1.6	0.3	2.2	13.8***
L x M	1	0.5	2.9	0.1	9.1**	0.4	1.5	4.4*
pH x L x M	4	0.2	0.5	0.1	1.0	1.7	0.2	4.4**

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 5.2. Regression models of soil microbial populations on pH

Dependent Variables	Soil type	Regression Equation	R square
Biomass C	High Non-rhizosphere	$Y=-0.996+0.313\times pH-0.023\times pH^2$	0.81***
	High Rhizosphere	$Y=-0.288+0.056\times pH$	0.89***
	Low Non-rhizosphere	$Y=-0.212+0.038\times pH$	0.76***
	Low Rhizosphere	$Y=-0.889+0.266\times pH+0.0068\times pH^2$	0.85***
Biomass N	High Non-rhizosphere	$Y=-100+25.2\times pH$	0.81***
	High Rhizosphere	$Y=-111+26.7\times pH$	0.88***
	Low Non-rhizosphere	$Y=-8.46+7.81\times pH$	0.50**
	Low Rhizosphere	$Y=-32.7+16.0\times pH$	0.44*
Rhizobia MPN	High Non-rhizosphere	$Y=-64.9+20.9\times pH-1.49\times pH^2$	0.91***
	High Rhizosphere	$Y=-39.7+15.5\times pH-1.22\times pH^2$	0.65**
	Low Non-rhizosphere	$Y=-84.9+27.0\times pH-1.94\times pH^2$	0.91***
	Low Rhizosphere	$Y=-119+39.4\times pH-2.98\times pH^2$	0.91***

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

Table 5.3. Pearson correlation coefficients between soil microbial populations and metal concentrations. N = 88 Prob > |r| under H0: Rho=0

Microbial Populations	Al	Ca	Cd	Mg	Mn	Zn	pH
Bacteria (rim)	-0.21*	0.37***	-0.19ns	0.26*	-0.39***	-0.21*	0.30**
Bacteria (r2a)	-0.25*	0.44***	-0.18ns	0.23ns	-0.41***	-0.21	0.36**
Actinomycetes	-0.11ns	0.32**	-0.23**	0.18ns	-0.39***	-0.27*	0.31**
Fungi	0.20ns	-0.23*	-0.02ns	-0.22*	0.16ns	0.14ns	-0.23*
Biomass C	-0.46***	0.74***	-0.62***	0.17ns	-0.86***	-0.72***	0.86***
Biomass N	0.21ns	-0.25*	-0.30*	-0.22ns	0.07ns	-0.16ns	-0.16ns
Rhizobium MPN	-0.41***	0.65***	-0.65***	0.16ns	-0.76***	-0.64***	0.69***

*, **, and *** indicate the significance at the 0.05, 0.01, and 0.001 probability levels, respectively.

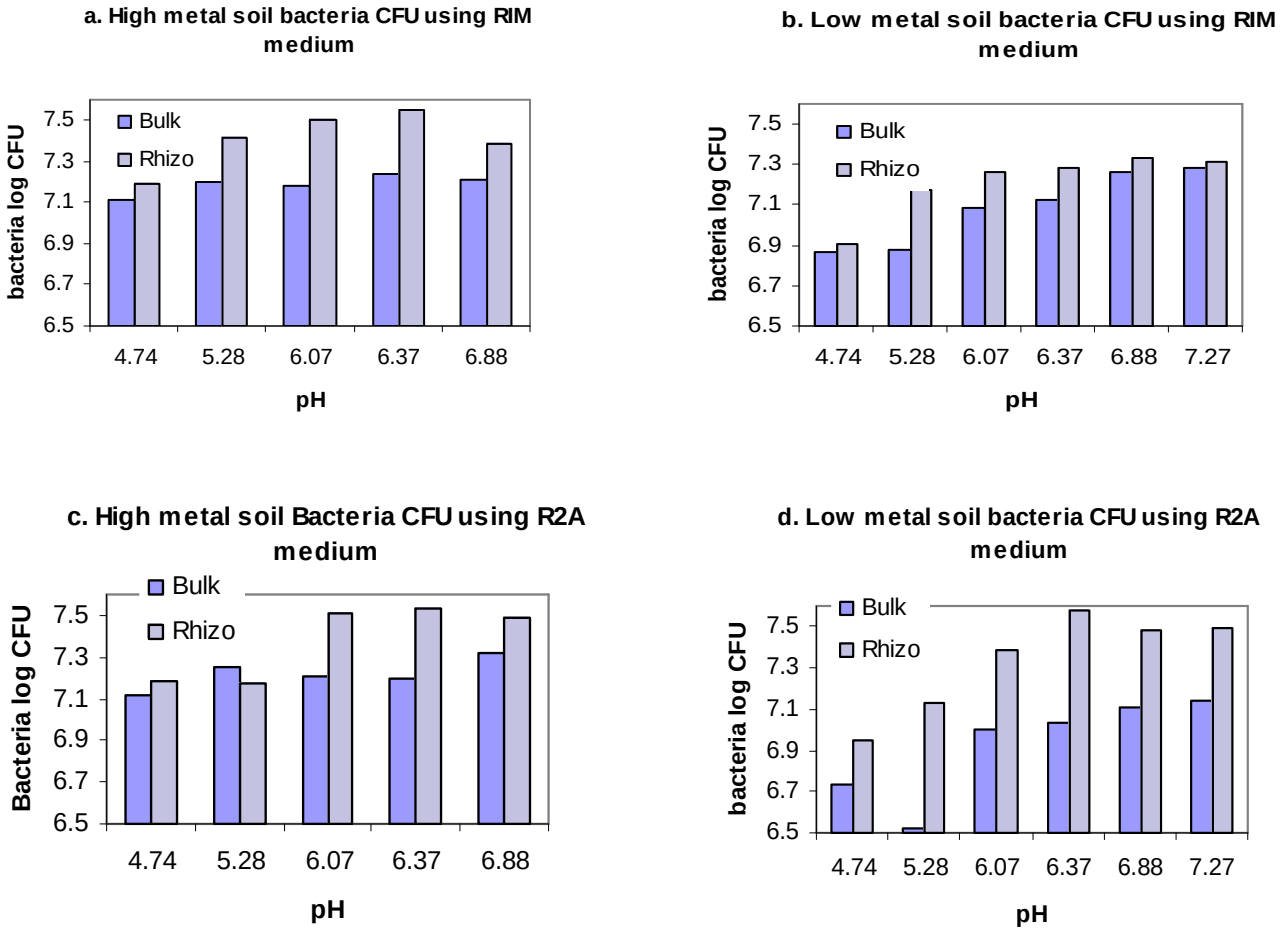

Figure 5.1. Soil pH effect on bacteria CFU in the high metal soil and low metal soil using RIM medium and R2A medium.

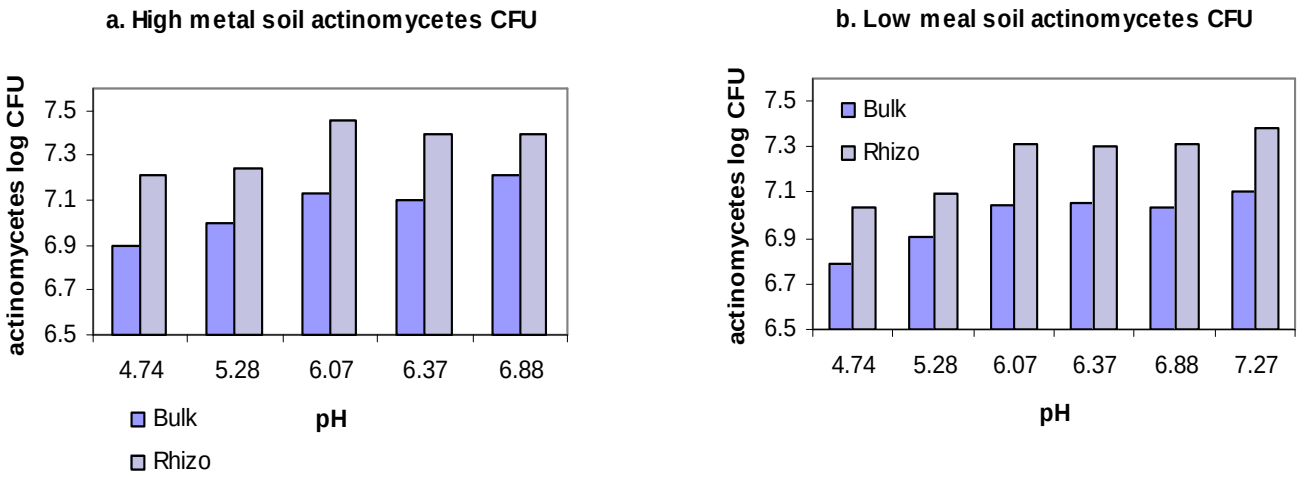

Figure 5.2. Soil pH effect on actinomycetes CFU in the high metal soil(a) and low metal soil(b).

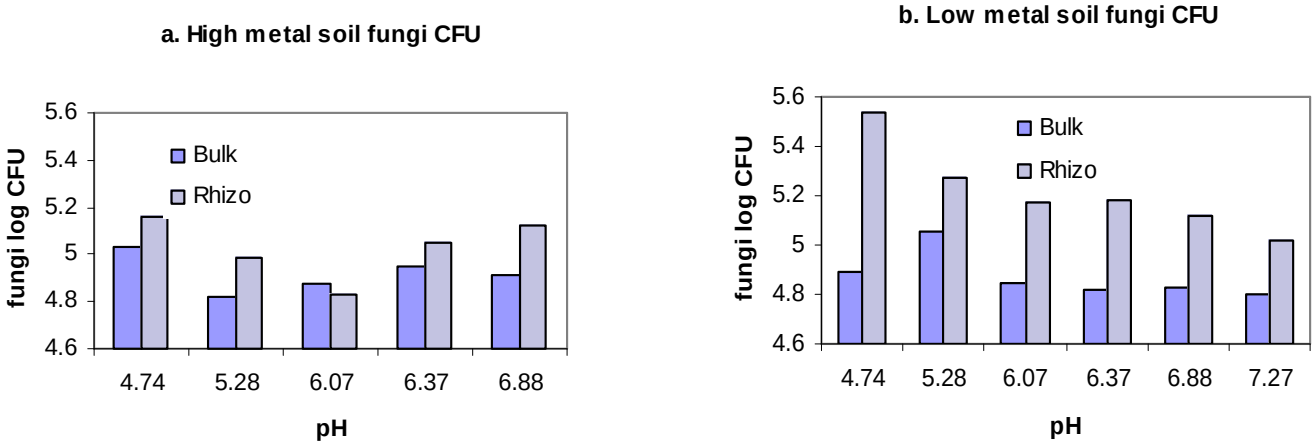

Figure 5.3. Soil pH effect on fungi CFU in the high metal soil (a) and low metal soil (b).

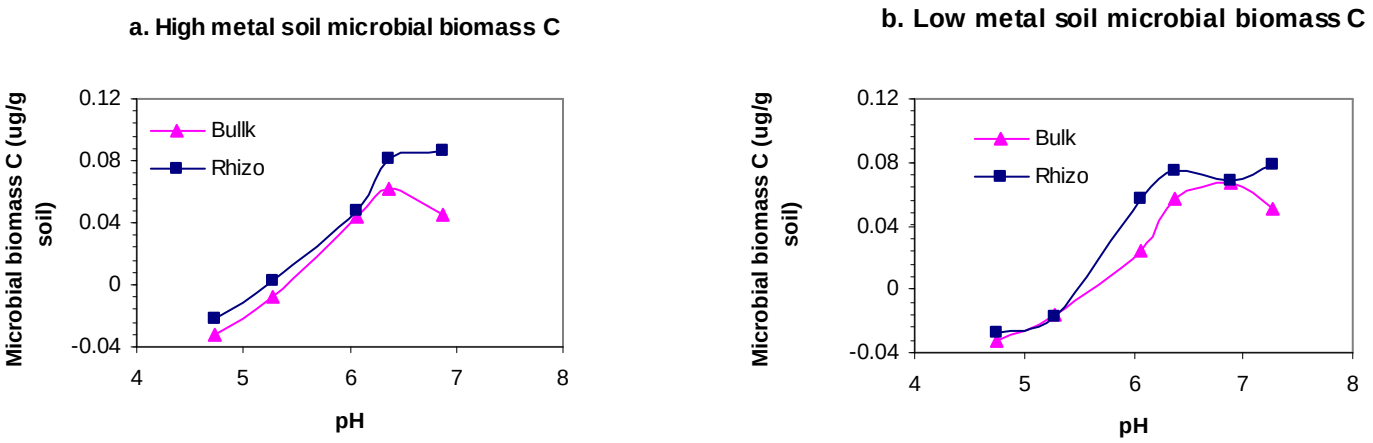

Figure 5.4. Soil pH effect on microbial biomass C in high metal soil (a) and low metal soil (b).

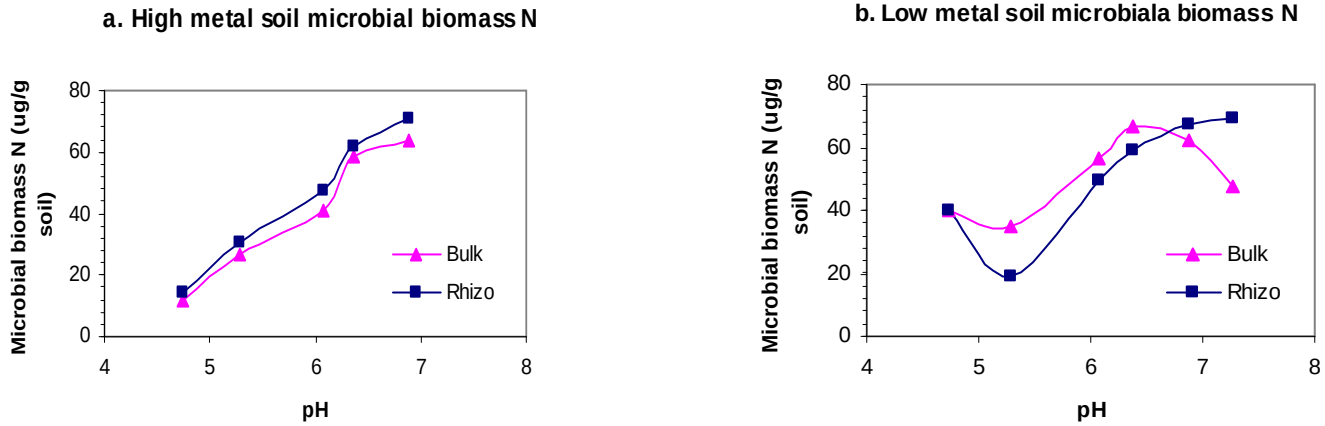

Figure 5.5. Soil pH effect on microbial biomass N in high metal soil (a) and low metal soil (b).

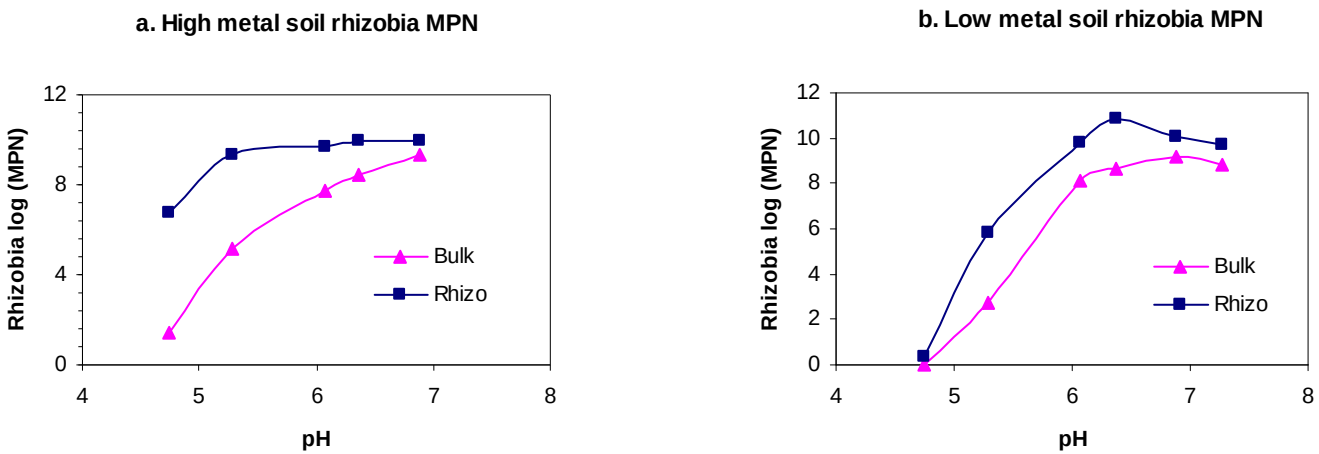

Figure 5.6. Soil pH effect on most probable number of white clover rhizobium in high metal soil (a) and low metal soil (b).

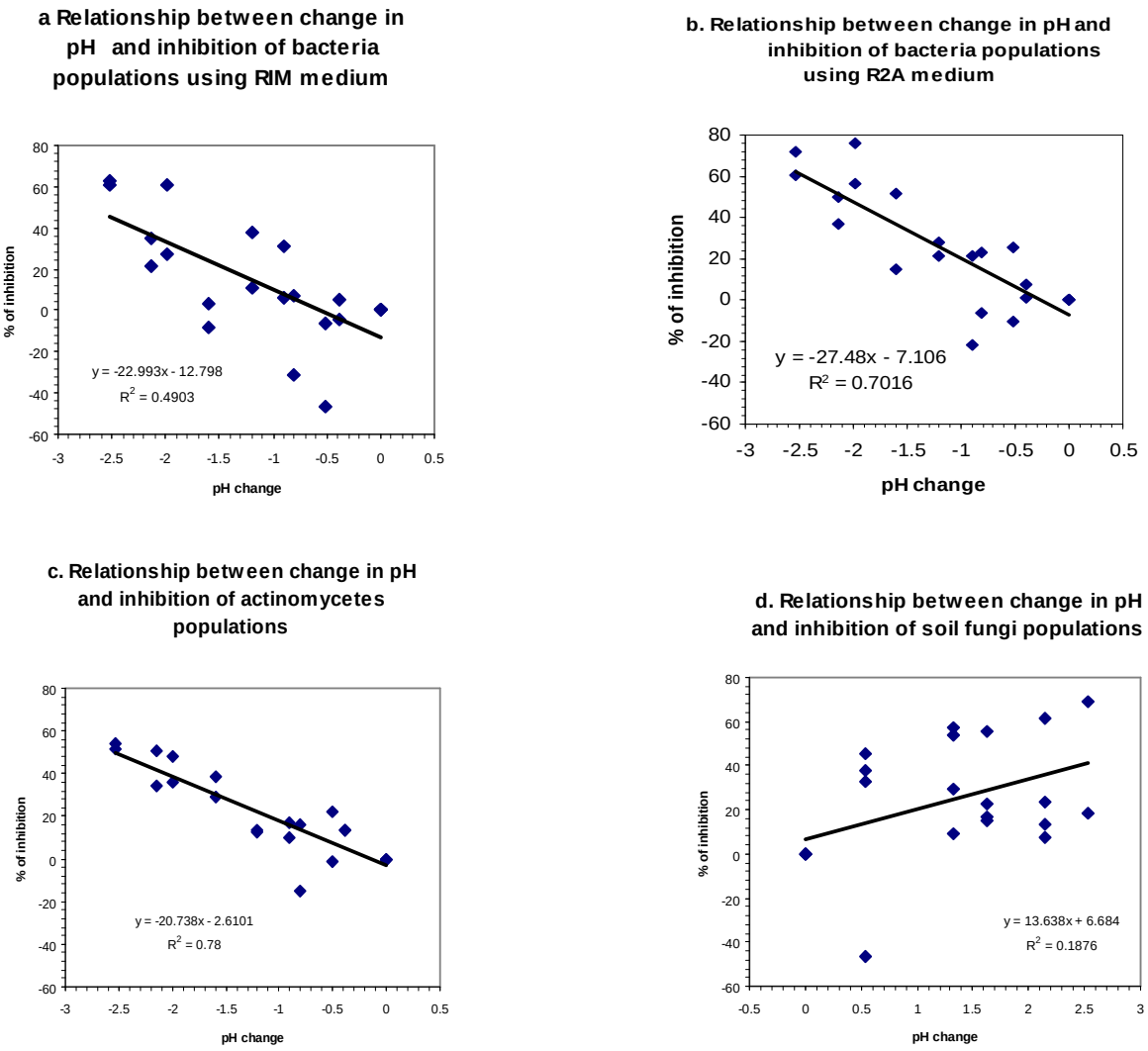

Figure 5.7. Inhibition effect of pH in soil microbial major group populations.

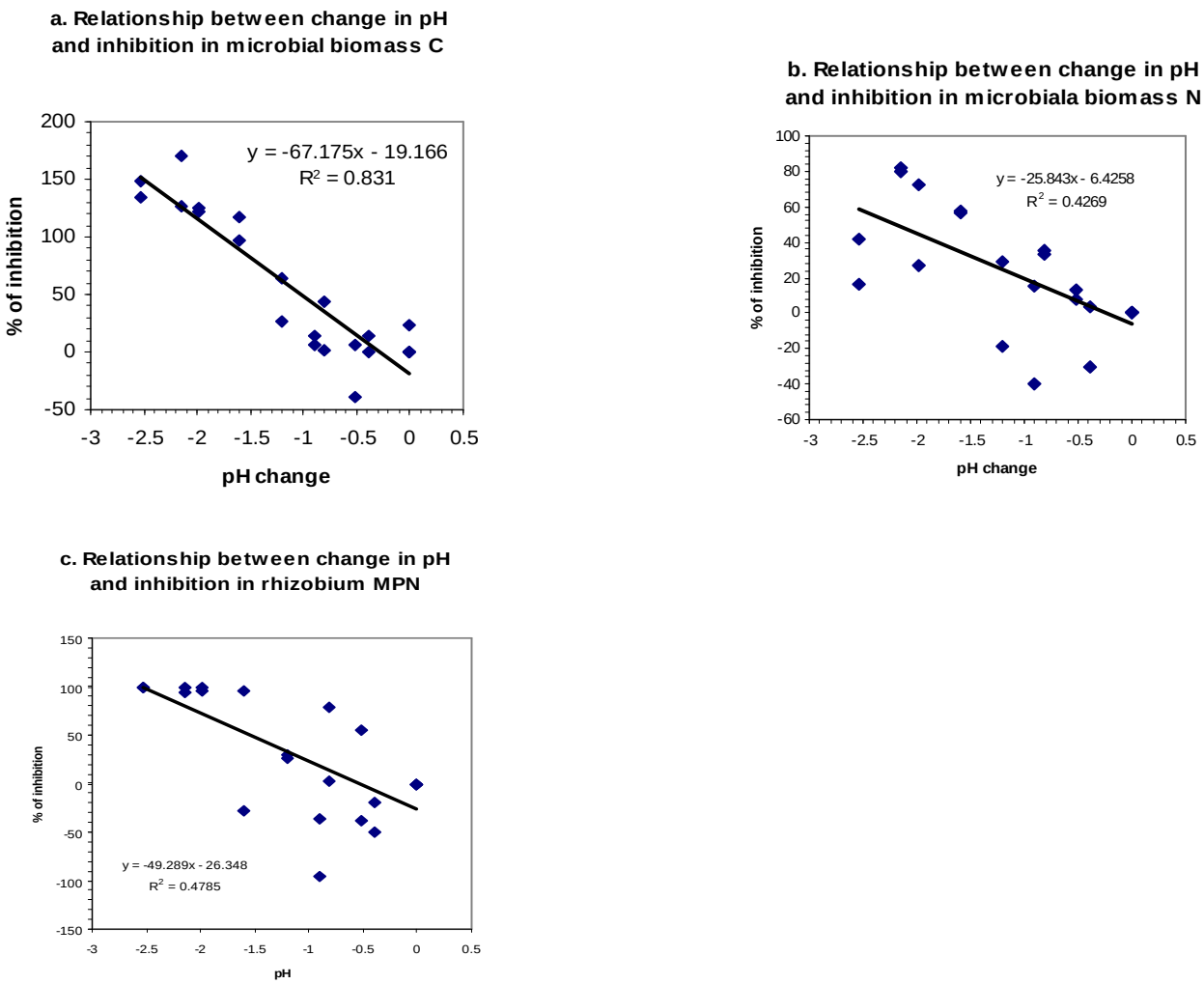

Figure 5.8. Inhibition effect of pH in soil microbial biomass C (a), N (b), and MPN estimation of rhizobium population (c)

Chapter 6: Ecological Risks of Reducing Soil pH during Zn and Cd Phytoextraction

ABSTRACT

Phytoextraction of soil Zn and Cd requires continual reduction in soil pH in order to maintain high metal uptake. Reducing pH of high metal soil, however, could negatively affect soil ecosystem function and health. The ultimate goal of soil remediation is to achieve a healthy soil ecosystem. However, no ecological risk assessment work currently exists to evaluate the suitability of reducing pH during phytoextraction from the ecology point of view. This work selected both the living organisms and microbial-mediated activities – two groups of variables to monitor the soil ecosystem health both before and after phytoextraction. Two Zn and Cd contaminated soils were adjusted to 5 or 6 different lower pH levels and *Thlaspi caerulescens* grown for 6 months. After phytoextracton, soil pH was re-adjusted to above 6.5 and incubated for 6 months. Soil enzyme activities, nitrification, respiration, number of bacteria, actinomycetes, fungi, and rhizobia were tested both under low pH treatments and after pH re-adjustment. Except acid phosphatase activity and the fungal population, reducing pH significantly reduced all tested activities and microbial populations. However, soil maintained partial resiliency even at the lowest pH treatment. After pH re-adjustment, the negatively impacted soil parameters were partially restored. Soil biological activities had a lower recovery rate than soil microbial populations. However, in the lowest pH treatment, none of these activities had returned to initial values prior to reducing pH. The threshold pH values were 6.1 and 5.3 for low and high metal soils, respectively. Above this value, most of the soil biological activities and all tested microbial populations returned to background levels within a short period.

Key words: Phytoextraction, pH, Soil ecosystem health, Disturbance

6.1 INTRODUCTION

Phytoremediation is defined as the use of plants to remove, contain, or render harmless contaminants in soils. Phytoextraction is a form of phytoremediation, which uses unusual hyperaccumulator plants to accumulate high quantities of metals in harvestable plant biomass. It offers a low cost strategy to clean up contaminated soils and the plant ash may also have economic value (Baker et al., 1994; Chaney et al., 2000). The hyperaccumulation process involves rapid uptake, high rates of translocation from roots to shoots, and huge storage capacity by vacuolar compartmentalization (Chaney et al., 1997). The availability of contaminant metals for plant uptake isa limiting factor of phytoextraction.

Phytoavailability of metals is strongly controlled by soil factors, such as pH, organic matter content, Fe and Mn oxides content, etc. Successful phytoextraction relies on appropriate soil and plant management practices to attain high yields and high metal concentrations in the plant biomass. Among the diverse strategies to enhance phytoextraction, pH adjustment has received the most attention, because heavy metals' phytoavailability is largely controlled by soil pH; lowering pH is expected to increase metal availability. Numerous studies have shown that lowering pH will result in desorption of heavy metals and increase plant available metal concentrations (Chlopecka et al., 1996; Cavallaro and McBride, 1980; Christensen, T.H. 1989; Harter, 1983). Studies conducted on other crops have also shown a negative correlation between soil pH and metal transferred to plants (Narwal et al., 1983; Castilho and Chardon, 1995). Only a few studies investigated the pH effect on *T. caerulescens* hyperaccumulation (Brown et al, 1994; Brown et al., 1995 b)

Although reducing soil pH appears to be an effective strategy to enhance phytoextraction of Cd and Zn, precaution is needed because low pH and elevated metal concentrations may cause negative impacts to already vulnerable soil ecological systems. Can phytoextraction be enhanced by lowering soil pH without creating a further threat to the environment? This question must be answered before further development and commercialization of this remediation technology proceeds.

Successful phytoextraction include not only metal removal from soil, but also return of a healthy soil ecosystem. One of the major misunderstandings during phytoextraction is the sole focus on a simple calculation of pollutant removal to determine the recovery of soil health. Almost all phytoextraction efficiency studies focus on the annual amount of metal extracted from soil. The subject of whether soil health is eventually recovered is largely ignored. Therefore, it is possible that the process of remediation itself could cause a more severe effect on soil ecology.

A healthy soil microbial community has two important characteristics: species richness and species evenness. Maintenance of viable, diverse and functioning soil microbial communities is essential to soil quality because most of the major processes in soil are carried out by soil microorganisms, such as C decomposition and nutrient cycling. Among soil microbes, bacteria, actinomycetes, and fungi are three of the important functional groups. Legumes and other non-leguminous N_2-fixation symbionts also have an important role in soil fertility and the global N-cycle.

Soil quality is defined as the capacity of soil to fulfill its unique ecosystem functions. Nutrient recycling is one of the vital functions performed by soil. As an integration of much soil environmental and biological information, soil microbial-mediated processes

are important and sensitive soil function indicators. Among them, acid phosphatase and alkaline phosphatase are important in the phosphorus cycle; Arylsulphatase activity is important in S cycling; nitrification is the soil microbial process in which ammonium (NH_4^+) is transformed into nitrate (NO_3^-). Heterotrophic CO_2 respiration is a key process regulating carbon cycling in the biosphere.

We selected both the living organisms and microbial-mediated activities – two groups of variables to monitor the soil ecosystem health both before and after phytoextraction. The hypothesis of this research is that low pH and high heavy metal concentrations have negative impacts on the soil microbial ecosystem, but these negative impacts can be eliminated or alleviated following an increase in soil pH once phytoextraction is complete.

6.2 MATERIALS AND METHODS

6.2.1 Site description and soil sampling

Soils samples were collected from the A horizon of two cultivated fields in the proximity of a former Zn smelter that had been in operation for nearly 100 years at Palmerton, PA. Metals released to the environment were primarily Zn and Cd resulting a metal concentration gradient according to the distance and direction from the smelter. We sampled two soils, one was about 4.5 km up wind from the smelter and characterized by relatively low metal concentrations. The other soil was about 1.4 km down wind from the smelter, and contained higher metals (Table 3.2). Both soils belong to Montevallo series (loamy-skeletal, mixed, subactive, thermic, shallow Typic Dystrudepts). Soils were first passed through a 1cm sieve to remove stones and large plant residues then passed through

a 4mm sieve. Soils were then homogenized and stored in closed containers to avoid dehydration.

6.2.2 Soil Characterization

Total Zn and Cd concentrations in soil were measured by extracting with concentrated hot nitric acid and measured by flame atomic absorption spectrometry. Soil particle size distribution was determined by the hydrometer method (University of Maryland, 1978). Soil pH was measured in a soil water suspension (10 g soil to 20 ml deionized water) after 1 h shaking at 180 rpm followed by 1 h standing. Organic matter content was determined by loss on ignition. Plant available Ca^{2+}, Mg^{2+}, and K^{+} were extracted with Mehlich (I) and determined with a Technicon Auto-Analyzer using a colorimeter for Mg and a flame photometer for K and Ca. Total N was determined by the combustion method. Plant available P was extracted with Mehlich (I) and analyzed with a Technicon Auto-Analyzer.

6.2.3 Soil pH adjustment and salt leaching

Different amounts of elemental sulfur (S) were used to adjust soil pH to desired levels based on a preliminary acid incubation experiment. Soil pH was monitored periodically by taking 10 g of soil and measuring the soil water suspension pH. Soil was thoroughly mixed every day to ensure equal distribution of sulfur and aerate the soil to speed up the S oxidation process. Completion of acidification was assumed when the same pH was measured for three consecutive weeks. Then 500 ml of deionized water was used to leach salt from each pot. After all water drained and there was no surface water

on the top of the soil, a second and third 500 ml increment of deionized water were used to repeat the process.

After harvest, soils were amended with different amounts of limestone [$Ca(OH)_2 \cdot 2H_2O$] to adjust the soil pH back to above 6.5 and followed by the same salt leaching process as described above. Then soils were incubated under room temperature for 6 months with regular watering to maintain adequate moisture.

6.2.4 Treatment structure and experimental design

A completely randomized block design with treatment in factorial combination was used with the following factors: 1) metal concentration (low and high), 2) presence of plant (w/ and w/o plant, i.e., rhizosphere soil and non-rhizosphere), and 3) soil pH (6.88, 6.37, 6.07, 5.28, 4.74). For the low metal soil, soil pH was adjusted to 6 levels; an additional pH treatment of 7.27 was used. There were 4 replications for each of the treatments which were randomly put into one of the four growth chambers.

6.2.5 Soil pH regime of acidification treatments and neutralization treatments

To avoid confusion, it is necessary to define several special terms used frequently in this paper. “Acidification” refers to the first pH adjustment, it is often interchangeably used with “the first pH adjustment” or “before pH re-adjustment”; “neutralization” refers to the second pH adjustment or pH re-adjustment, i.e., the reduced pH treatments were increased by adding limestone. Each pH treatment number denotes two unique pH values both in acidification and neutralization treatments as shown in Table 6.1. For

convenience, in the following discussion, we used the treatment number to refer to pH values in some cases.

6.2.6 Soil biological activities

Soil enzyme activities were measured by the colorimetric determination of p-nitrophenol released by referring to a calibration standard curve (Tabatabai 1994). For each soil sample, controls were also examined, and the p-nitrophenol concentration was subtracted from the sample's value. Triplicate samples were conducted for each soil sample.

Soil nitrification potential was measured by the shaken soil-slurry method (Hart et al., 1994). Ten gram of soil was mixed with 80 ml of a nitrification substrate solution mixture and shaked in a reciprocal shaker at 300 rpm for 48 h. A portion of the soil slurries was sampled at 6, 12, 18, 24, 36, and 48 h. Slurry were then centrifuged and filtered using Whatman no. 40 filter paper. The nitrate in the solution was analyzed by a colorimetric method. The rate of nitrate production was then calculated by linear regression of these results.

Soil respiration was determined by closed jar incubation with NaOH trap and followed by acid titration (Zibilske, 1994).

6.2.7 Plate counting of bacteria, actinomycetes, and fungi

Enumeration of viable bacteria, actinomycetes, and fungi in soils were performed by the spread plate technique with different culture media. Ten gram of moist soil was added to 95 ml of sterile deionized water and mixed for 1 min at 22000 rpm in a Waring blender.

After settling for 1 min, 10-fold dilutions were made using sterile deionized water. Then 0.1 ml aliquots from each of the appropriate dilutions were transferred to the agar plates. Three replicate plates were inoculated at each given dilution. The inoculated plates were then inverted and incubated in a dark incubator with temperature set at 28^{o}C. The number of bacteria, actinomycetes, and fungi were counted after 4, 4, 5 days of incubation, respectively.

The media used for culturing of viable bacteria were R2A (Reasoner and Geldreich, 1985) and RIM. Starch casein medium (Küster and Williams, 1964; Wellington and Toth, 1994) and Martin's medium (Wollum, 1982; Parkinson, 1994) were used or culturing of actinomycetes and fungi, respectively.

6.2.8 Estimation of population size of the indigenous *R. leguminosarum* bv. *trifolii*

The numbers of rhizobia able to nodulate white clover were determined by the most probable number (MPN) method (Weaver and Graham, 1994) using a 10-fold soil dilution series. *Trifolium repens* (white clover cv. Menna) was used as the trap host plants for *R. leguminosarum* bv. *trifolii*. Five replicate plant infection tubes were inoculated with 1 ml aliquots at each dilution step and the tubes were placed in a controlled environment growth chamber. The chamber was set at 12h/12h day/night cycles. Temperature was maintained at 28^{o}C during the day period and 24^{o}C during the night. Light intensity was above 400 µmol photon m^{-2} s^{-1}; relative humidity was 65%. Five replicate tubes inoculated with *R. leguminosarum* bv. *trifolii* USDA 2055 obtained from the USDA *Rhizobium* Germplasm Collection at Beltsville, MD were performed as positive controls for each set of samples, as well as five replicate tubes inoculated with

sterile deionized water as negative controls. Nodulation was assessed after 3 wks. Numbers of rhizobium were calculated using the MPNES computer program (Woomer et al., 1990; Woomer, 1994).

6.2.9 Statistical Analysis

Statistical analysis was conducted using SAS version 8.2 (SAS Institute, 2001). The assumption of normality was tested by examining the plot of residuals and calculating the Shapiro-Wilk statistic. The homogeneity of variance was tested by examining a plot of predicted values versus residual values. The Spearman test was used to test the correlation between the predicted value and absolute value of the residue. Logarithm transformation of data was performed for some variables when needed. After checking that data met the assumptions, the PROC MIXED procedure was used for univariate ANOVA to determine the main factor and interaction effect with block as a random factor. When significant effects were detected, pair-wise treatment mean comparisons were made using a Least Significance Difference (LSD) t-test on pH treatment means. Differences between means of acidification and neutralization adjustments were compared by a paired t-test. Unless otherwise indicated, the statistical significance level was set as $p \leq 0.05$.

6.3 RESULTS

6.3.1 Acid phosphatase activity (AcP)

Reducing soil pH significantly increased this enzyme activity (Table 6.2). For both high and low metal soils, each lower pH treatment had significantly higher AcP activity

than its previous higher pH treatment. After pH increased to above 6.5, enzyme activity decreased. The lower the pH before neutralization, the greater was activity reduction after pH neutralization in the high metal soil. This was also true for low metal soil pH treatments 7.27, 6.88, 6.37 and 6.07. At pH 5.27, activity reduction due to the pH increase was relatively small. At pH 4.74, activity increased after pH was increased to 6.76. For both soils, increasing pH did not return the acid phosphatase activity to its original level. The pH treatments of 6.07, 5.27, and 4.74 still had significantly higher activity than pH treatments of 6.88 and 6.37 in the high metal soil. This was even more obvious in the low metal soil.

6.3.2 Alkaline phosphatase activity (AlP)

Reducing pH significantly reduced AlP activity. After pH was increased to above 6.5, AlP activity did not recover back to its original level (Table 6.3). For high metal soil, AlP activity showed a slight decrease even after pH neutralization in treatments 6.37, 6.07, and 5.27. Only at pH 5.27 did activity increase after pH neutralization. For the low metal soil, responses to pH neutralization were variable. Activity was further reduced despite the pH increase in treatments 6.88, 6.37, and 4.74. Only in treatments 6.07 and 5.27, did pH neutralization adjustment increase AlP activity. Except for pH 4.74 in the low metal soil, there was no significant difference in AlP activity between acidification and neutralization adjustments.

6.3.3 Arylsulphatase activity

Reducing pH significantly reduced arylsulphatase activity.However, a mong the three tested enzyme activities, arylsulphatase activity had the best recovery after pH re-adjustment (Table 6.4). Activity was increased at all pH treatments. Significant increases occurred at pH treatments 6.37 and 4.74 in the high metal soil, and 6.07, 5.27, and 4.74 in the low metal soil. In high metal soil pH 6.37, pH re-adjustment fully restored arylsulphatase activity. The same effect was noted in the low metal soil treatment 6.07. Except for these changes, there were still significant differences between pH treatments. Previous lower pH treatments usually had significantly lower activity than the higher pH treatments.

6.3.4 Soil respiration

Reducing pH significantly lowered soil respiration. After pH re-adjustment, respiration increased in pH treatments of 6.07 and 4.74 in the high metal soil while decreased in the treatments 6.37 and 5.27. Increased pH did not cause any significant difference, and the lowest pH treatment still had significantly lower respiration than other pH treatments. In the low metal soil, increased pH partially restored soil respiration in all pH treatments. This was especially apparent in the lowest pH treatment, where respiration increased from 0.43 $\mu g\ kg^{-1}\ h^{-1}$ to 0.61 $\mu g\ kg^{-1}\ h^{-1}$ (Table 6.5).

6.3.5 Nitrification potential

Soil nitrification potential was significantly reduced by acidification, but showed variable responses to pH re-adjustment (Table 6.6). In the high metal soil, nitrification

was further reduced in the pH treatments of 6.37 and 6.07 while increased in the pH treatments of 5.27 and 4.74. For the low metal soil, nitrification was reduced at pH treatments of 6.37 and 6.07, while nitrification increased in the pH treatments of 6.88, 5.27 and 4.74. The negative values in the lowest pH treatments of low metal soil disappeared. But increasing pH did not cause significant changes. The lowest pH treatments still had a significantly lower rate of nitrification than the highest pH treatment in both soils.

6.3.6 Viable bacteria using R2A medium

The number of bacteria using R2A medium was significantly affected by pH. It tended to decrease with reduced pH (Table 6.7). The highest numbers of bacteria appeared at the higher pH treatments. But there was no significant difference between all five pH treatments in the high metal soil. For low metal soil, bacterial numbers were significantly reduced when pH was reduced to 5.27. After pH readjusted, the number of bacteria slightly increased in most pH treatments. The increase was significant for the low metal soil treatment 5.27. The low metal soil, treatment 4.74, also showed a large increase. However, the number of bacteria in the lowest pH treatment was still significantly lower than the highest pH treatment in both soils.

6.3.7 Viable bacteria using RIM medium

The number of bacteria using RIM medium tended to decrease with reduced pH (Table 6.8). For the high metal soil, there was no significant difference between all pH treatments. For the low metal soil, there was no significant difference between the three

highest pH treatments. But at pH 6.07, bacterial numbers were significantly reduced. At pH 5.28 and 4.74, a further significant reduction occurred. After soil pH re-adjustment, bacterial numbers showed different degrees of recovery at all pH treatments. For high metal soil, the lower pH treatments all had higher number of bacteria than the control treatment. For the low metal soil, only the lowest pH treatment still had significantly lower numbers of bacteria than the control soil. At other pH treatments, bacterial numbers were fully restored.

6.3.8 Viable actinomycetes using Starch Casein medium

Numbers of viable actinomycetes in soil were significantly affected by pH. Numbers tended to decrease with reduced pH (Table 6.9). For high metal soil, the three higher pH treatments had significantly greater numbers of actinomycetes than the two lowerest pH treatments. For low metal soil, the four higher pH treatments had significantly higher numbers of actinomycetes than the two lowest pH treatments. pHre -adjustment increased the number of actinomycetes in all treatments. However, this increase was not enough to fully restore actinomycete numbers. The two lowest pH treatments still had significantly lower numbers than the control soil.

6.3.9 Viable fungi using Martin's medium

The number of fungi was significantly influenced by soil acidification. Numbers of fungi increased with reduced pH (Table 6.10). Highest numbers generally occurred at the lowest pH treatments. In the high metal soil, increasing pH generally decreased the number of fungi. After pH re-adjustment, all pH treatments were statistically similar to

the control soil. For low metal soil, however, the number of fungi increased due to pH re-adjustment except for the lowest pH treatment. The lower pH after acidification, the smaller the increase after pH re-adjustment.

6.3.10 Population size of the indigenous *R. leguminosarum* bv. *trifolii*

The number of indigenous rhizobia in the soil was influenced by pH. With decreasing pH during phytoextraction, the number of rhizobia were drastically reduced, even totally eliminated (Table 6.11). For high metal bulk soil, a significant reduction was observed at pH 6.07, then when pH was further reduced to 5.28 and 4.74, numbers were further significantly reduced. For low metal soil, from pH 7.27 to 6.07, rhizobia numbers were relatively constant. However, below pH 6.07, numbers rapidly decreased with decreasing of pH and reached zero at the lowest pH treatment. pH re-adjustment greatly increased the number of rhizobia in the high metal soil in all pH treatments. Rhizobia numbers increased ten-fold in the pH treatment 6.37 and increased more than 2 magnitude in the pH treatments 6.07, 5.27, and 4.74. In the low metal soil, rhizobia numbers slightly decreased at pH treatments of 6.88, 6.07, and 5.27. Notably, at the lowest pH treatment, re-adjustment of pH caused in the “re-appearance” of rhizobia in soil. Despite these changes, the lower pH treatments still had significantly lower number of rhizobia than the higher pH treatments.

6.3.11 0.1M $Sr(NO_3)_2$ extractable Cd, Zn, Al, Mn, Ca, and Mg

The concentration of 0.1 M $Sr(NO_3)_2$ extractable Cd was significantly affected by pH. For the high metal soil, Cd concentration was significantly increased with each

reduction in pH in the acidification adjustment. For low metal soil, starting from pH 6.07, each lower pH treatment significantly increased extractable Cd concentrations compared to the next higher pH treatment. After pH re-adjustment, Cd concentrations decreased with increasing pH (Table 6.12). For high metal soil, pH treatments of 6.37 and 6.07 contained similar Cd concentrations to the control soil. pH treatments 5.27 and 4.74 showed a very significant decline. For low metal soil, all pH treatments had statistically similar Cd concentration as the control soil.

Similarly, reducing pH significantly increased Zn concentrations. For the high metal soil, extractable Zn concentrations were significantly increased with each reduction in pH during the acidification adjustment. For low metal soil, starting from pH 6.07, each lower pH treatment significantly increased Zn concentration compared to the next higher pH treatment. After pH re-adjustment, Zn concentrations decreased with increasing pH (Table 6.13). Except for pH treatments of 6.88 and 6.37 in the low metal soil, pH re-adjustment caused a significant decline in Zn concentration at all pH levels. Zn concentrations decreased by 80% in the pH treatment 4.74 of high metal soil and 96% in the low metal soil.

Reducing pH significantly increased extractable Al concentrations in both soils. The response of Al concentration to pH, however, was not the same for the two soils. For high metal soil, from the highest pH to the lowest, Al increased byabout 30%. However, for low metal soil, there was 8 to 11 fold increase. The final concentration in the low metal soil reached 71.8 mg kg^{-1}. After pH re-adjustment, extractable Al generally decreased to near background levels (Table 6.14). In the low metal soil, Al concentrations decreased at all pH treatments to even less than the control.

Mn responded to pH changes similar to that of Al. Concentrations were greatly increased with decreasing pH. In both soils, each lower pH treatment had a significantly higher Mn concentration than its previous higher pH treatment. After pH neutralization adjustment, Mn concentration was significantly reduced in most pH treatments (Table 6.15). Extractable Mn concentrations decreased by 71%, from 22.7 to 6.5 mg kg^{-1} in the high metal soil pH treatment 4.74 and decreased by 88%, from 24.6 to 3.0 mg kg^{-1} in the low metal soil.

The pattern of changes in Ca was opposite to that of Cd, Zn, Mn, and Al. Calcium concentration decreased with decreasing pH. For high metal soil, there was no significant difference between the first three higher pH treatments. Above pH 6.07, each lower pH treatment significantly reduced Ca concentration compared to its previous higher pH treatment. pH re-adjustment resulted in a significant increase in Ca concentrations for most pH treatments (Table 6.16). All pH treatments in the high metal soil recovered to the control soil Ca concentration levels.

Mg had a unique pattern responding to pH change. Reducing pH significantly reduced Mg concentration, which was similar to Ca. However, pH neutralization adjustment did not cause Mg concentration to be restored to original levels. Instead, concentrations of Mg were further reduced (Table 6.17).

6.4 DISCUSSION

It is important to assess whether the negative impacts due to reducing pH to improve phytoextraction can be eliminated or alleviated following an increase in the soil pH once phytoextraction is complete. As soil remediation often found it difficult to define “How

clean is clean", regarding risk assessment work, we often face a similar question "How large a risk is acceptable?" It is hard to define a mathematic boundary. The ultimate goal of soil remediation of any kind is to restore a healthy soil ecosystem. Soil health and soil quality are often used interchangeably with slightly different emphasis. Definitions of soil quality often vary among authors. Recently, Karlen et al. (1997) define"soil quality is the fitness of a specific kind of soil to function within its capacity and within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation." Resiliency is the ability to recover quickly to conditions and relationships existing prior to the disturbance (Holling 1973). A healthy ecosystem can retain its ability for self-organization and allow the maintenance of desired conditions over time. Therefore, in the present research, we evaluated the acidification risk in high metal soils from two aspects, 1) whether a specific pH caused irreversible soil quality degradation of the Zn and Cd contaminated soils, i.e., the disturbance was so abrupt that soil lost its resiliency even after the disturbance failed to exist; and 2) In the case where soil retained its resiliency, to what degree was recovery possible?

Among the ten responsive variables measured, two needed special attention: acid phosphatase activity and the fungal population. Apparently reducing pH favored their activity and increased population density. As Vogl (1983) stated that contrary to equilibrium theory, "Many organisms exist because of certain catastrophic factors or extreme conditions, and not in spite of them." Therefore neutralizing the reduced pH decreased activities but they were still higher than background levels. In these cases, reducing pH was a "good" disturbance and did not pose any risk to ecosystem function,

rather, it had the potential to enhance species and functional diversity and hence contribute to ecosystem health.

All other variables had different degrees of recovery after soil pH re-adjustment. For low metal soil, there was no significant difference in alkaline phosphatase activity between before and after pH re-adjustments except at the lowest pH treatment where activity was significantly further reduced. Arylsulphatase activity had the best recovery after pH re-adjustment. Activity was increased in all pH treatments. Increase in pH also partially recovered soil respiration in all pH treatments. Especially in the lowest pH treatment, respiration showed a drastic increase. Nitrification had poor recovery but the previous negative values in the lowest pH treatments disappeared. This indicated soil had partial resiliency even at the lowest pH treatment. More important, however, none of these activities had returned to its initial values prior to reducing pH. The first significant reduction occurred at pH treatment 4, 5, 2, 3 for AlP, arylsulphatase, respiration, and nitrification, respectively. Soil biological activities apparently had lower recovery rate than soil microbial populations. For the latter, the first significant reduction occurred at pH treatment 5, 6, 5, 4 for Br2a, Brim, actinomycete, and rhizobia populations counts, respectively. It seemed that 6.07 could be the pH value of concern for the low metal soil. Above this value, most of the soil biological activities and all tested microbial population returned to background levels within a short period, i.e., 6 month, after soil pH being re-adjusted to above 6.5.

For the high metal soil, the significant reduction occurred at pH treatment 2, 3, 5, 5 for alkaline phosphatase, arylsulphatase, respiration, and nitrification, respectively. And for soil microbial populations, the first significant reduction occurred at pH treatment 5, 4,

4 for Br2a, actinomycete, and rhizobia populations counts, respectively. There was no reduction of Brim at all pH treatment. Therefore, 5.27 could be the pH value of concern for the high metal soil. Above this value, mostsoil biological activities and all tested microbial population return to background levels within a short period after soil pH re-adjustment to above 6.5. Note that the value of pH treatment 4 in low metal soil was 6.07 while in high metal soil it was 5.27. High metal soil appeared to have better resiliency regarding lowering pH and could tolerant lower pH. This observation suggests that ecosystems are not equally resilient and will not respond uniformly to a particular disturbance.

The pH threshold for possibly irreversible damage was around 6.1 for low metal soil, and around 5.3 for high metal soil. These results were based on short-term disturbance response observations. If soil is under continual recovery, it is possible that soils receiving lower pH treatments may also be able to continue to recover toward background levels over extended time. However, it is also possible that the soils may not fully recover even over a prolonged period. Longer term studies are needed.

Despite these on-site impacts, reducing pH also caused off-site impacts, mainly potential metal leaching problems because more metals were present in the soil solution. About 2.0 and 0.7 mg kg^{-1} Cd was leached out of soil in the lowest pH treatment from the high metal soil and low metal soil, respectively. About 216 and 76 mg kg^{-1} of total Zn was lost at the lowest pH treatment. Leaching problem was mush less severe if pH were maintained at or above threshold values. Only about 0.19 and 0.16 mg kg^{-1} Cd was leached out of soil in the pH treatment 4 from the high metal soil and low metal soil, respectively. And only 59 and 4 mg kg^{-1} Zn was leached out of soil in the pH treatment 4

from the high metal soil and low metal soil, respectively. For rhizosphere soil the leaching problem was negligible. There was no leaching of Cd in all pH treatments, and only 26 mg kg^{-1} was leached in the high metal soil pH treatment 4 and no leaching in the low metal soil up to pH treatment 4. Data on the analysis of 0.1M $Sr(NO_3)_2$ extractable Cd, Zn, Al, Mn showed that after pH re-adjustment, metal concentrations were markedly reduced to such a level that they would not present a concern.

6.5 CONCLUSIONS

1) Except for acid phosphatase activity and the fungal population, reducing pH significantly reduced all tested activities and microbial populations.
2) Soil retained the capacity to recover toward the condition before acidificataion treatment even at the lowest pH treatment.
3) Soil biological activities had lower recovery rate than soil microbial populations after pH re-adjustment. However, full recovery may take a much longer time and may be very difficult to achieve original levels seen in the lowest pH treatment.
4) It is recommendable to keep the pH values above 6.1 and 5.3 for low and high metal soils, respectively. Above this value, most of the soil biological activities and all tested microbial populations can return to background levels within a short period. Moreover, metal leaching problem was negligible if soil pH were maintained at above these values.

Table 6.1. Soil pH regime of acidification and neutralization adjustments

Metal	pH treatment number	Acidification adjustment	Neutralization adjustment
High	1	6.88[†]	7.15
	2	6.37[‡]	7.13
	3	6.07	7.15
	4	5.27	6.77
	5	4.74	6.76
Low	1	7.27[¶]	7.39
	2	6.88	7.21
	3	6.37	7.01
	4	6.07	6.92
	5	5.27	6.88
	6	4.74	6.62

[†] pH treatment number 1 is the control soil pH, note it had a slight increase after incubation in both soils.
[‡] Means of four blocks.

Table 6.2
Comparison of means (SD) for acid phosphatase activity after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between acid phosphatase activity after acidification and neutralization adjustments are calculated using logarithm transformed data, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively (paired t-test)

Metal	pH trt.	p-nitrophenol conc. (mg kg^{-1} h^{-1}) Neutralization	p-nitrophenol conc. (mg kg^{-1} h^{-1}) Acidification	Difference (neutra – acidi)
High	1	312 (10.4) [b]	312[†] (10.4) [e]	0
High	2	344 (20.5) [b]	335 (19.6) [d]	9.00
High	3	331 (19.9) [b]	375 (20.5) [c]	-44.0
High	4	407 (33.2) [a]	531 (31.0) [b]	-124
High	5	425 (11.9) [a]	716 (26.0) [a]	-291**
Low	1	260 (3.47) [d]	260 (3.48) [e]	0
Low	2	356 (19.1) [c]	374 (11.6) [d]	-18.0
Low	3	404 (26.4) [cb]	506 (16.2) [c]	-102
Low	4	438 (12.1) [b]	664 (21.7) [b]	-226***
Low	5	753 (23.9) [a]	899 (43.2) [b]	-146
Low	6	791 (32.7) [a]	726 (60.7) [a]	65.0

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.3
Comparison of means (SD) for alkaline phosphatase activity after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between alkaline phosphatase activity after acidification and neutralization adjustments are calculated using logarithm transformed data, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively (paired t-test)

Metal	pH trt.	p-nitrophenol conc. (mg kg^{-1} h^{-1}) Neutralization	p-nitrophenol conc. (mg kg^{-1} h^{-1}) Acidification	Difference (neutra – acidi)
High	1	447 (26.4) ^{d}	447† (26.4) ^{a}	0
High	2	424 (31.8) ^{c}	432 (12.4) ^{a}	-8.00
High	3	406 (38.2) ^{b}	421 (39.6) ^{a}	-15.0
High	4	249 (43.1) ^{ab}	288 (23.8) ^{b}	-39.0
High	5	165 (6.18) ^{a}	147 (12.5) ^{c}	18.0
Low	1	444 (33.6) ^{a}	444 (33.5) ^{a}	0
Low	2	415 (31.8) ^{a}	467 (43.6) ^{a}	-52.0
Low	3	437 (35.6) ^{a}	486 (46.3) ^{a}	-49.0
Low	4	344 (29.3) ^{b}	302 (26.6) ^{b}	42.0
Low	5	139 (13.4) ^{c}	137 (16.7) ^{c}	2.00
Low	6	82.1 (11.6) ^{d}	131 (14.2) ^{c}	-48.9*

† Acidification values were adjusted by a ratio based on the control soil values.

Table 6.4
Comparison of means (SD) for arysulphatase phosphatase activity after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between arylsulphatase activity after acidification and neutralization adjustments are calculated using logarithm transformed data, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively (paired t-test)

Metal	pH trt.	p-nitrophenol conc. (mg kg^{-1} h^{-1}) Neutralization	p-nitrophenol conc. (mg kg^{-1} h^{-1}) Acidification	Difference (neutra – acidi)
High	1	189 (14.8) [a]	189[†] (14.8) [a]	0
High	2	175 (12.2) [a]	149 (12.9) [b]	26.0*
High	3	130 (8.41) [b]	99.4 (10.7) [c]	30.6
High	4	93.2 (18.8) [c]	56.6 (6.45) [d]	36.6
High	5	48.3 (5.23) [d]	25.3 (3.23) [e]	23.0*
Low	1	176 (12.9) [a]	176 (12.9) [a]	0
Low	2	173 (10.8) [a]	169 (12.4) [a]	4.00
Low	3	197 (13.7) [a]	164 (14.1) [a]	33.0
Low	4	175 (12.5) [a]	116 (8.59) [b]	59.0*
Low	5	84.9 (6.07) [b]	36.0(3.56) [c]	48.9***
Low	6	43.7 (4.94) [c]	14.3 (2.48) [d]	29.4*

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.5
Comparison of means (SD) for soil respiration after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between soil respiration after acidification and neutralization adjustments are analyzed using t-test, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively.

Metal	pH trt.	CO_2 ($\mu g\ kg^{-1}\ h^{-1}$) Neutralization	CO_2 ($\mu g\ kg^{-1}\ h^{-1}$) Acidification	Difference (neutral – acidi)
High	1	0.76 (0.004) [a]	0.76[†] (0.005) [a]	0
High	2	0.69 (0.05) [a]	0.73 (0.15) [a]	-0.04
High	3	0.73 (0.08) [a]	0.61 (0.08) [ab]	0.12
High	4	0.65 (0.05) [a]	0.79 (0.16) [a]	-0.14
High	5	0.52 (0.05) [b]	0.48 (0.07) [b]	0.04
Low	1	1.06 (0.05) [a]	1.07 (0.05) [a]	0.01
Low	2	0.76 (0.06) [b]	0.69 (0.05) [b]	0.07
Low	3	0.71 (0.07) [bc]	0.67 (0.06) [b]	0.04
Low	4	0.71 (0.12) [bc]	0.61 (0.07) [bc]	0.10
Low	5	0.69 (0.02) [bc]	0.61(0.08) [bc]	0.08
Low	6	0.61 (0.06) [c]	0.43 (0.08) [c]	0.18

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.6
Comparison of means (SD) for soil nitrification potential after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between soil nitrification rate after acidification and neutralization adjustments are analyzed using t-test, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively.

Metal	pH trt.	NO_3^- (ug kg^{-1} h^{-1}) Neutralization	NO_3^- (ug kg^{-1} h^{-1}) Acidification	Difference (neutra – acidi)
High	1	56.8 (9.21) [a]	56.8[†] (9.21) [a]	0
High	2	47.5 (16.8) [ab]	66.4 (20.1) [a]	-18.9
High	3	40.8 (15.7) [ab]	45.6 (7.58) [a]	-4.8
High	4	48.7 (7.15) [ab]	10.5 (14.8) [b]	38.2
High	5	16.2 (11.4) [b]	5.23 (3.53) [b]	11.0
Low	1	60.6 (14.5) [ab]	60.6 (14.5) [a]	0
Low	2	74.7 (12.0) [a]	48.5 (12.1) [a]	26.2
Low	3	32.1 (11.6) [bc]	43.6 (7.98) [a]	-11.5
Low	4	6.81 (13.0) [dc]	35.9 (2.17) [b]	-29.1
Low	5	9.32 (9.20) [d]	-3.51(12.7) [c]	12.8
Low	6	7.07 (4.86) [dc]	-14.7 (26.8) [d]	21.8

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.7
Comparison of means (SD) for soil viable bacteria using R2A medium (Br2a) after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between Br2a after acidification and neutralization adjustments are calculated using logarithm transformed data, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively (paired t-test)

Metal	pH trt.	Br2a (Log CFU) Neutralization	Br2a (Log CFU) Acidification	Difference (neutra – acidi)
High	1	7.07 (0.01) [a]	7.07[†] (0.01) [a]	0
High	2	6.96 (0.03) [ab]	6.95 (0.15) [a]	0.01
High	3	7.01 (0.03) [ab]	6.96 (0.03) [a]	0.05
High	4	6.94 (0.03) [ab]	7.01 (0.06) [a]	-0.07
High	5	6.87 (0.11) [b]	6.88 (0.04) [a]	-0.01
Low	1	6.33 (0.05) [a]	6.33 (0.05) [a]	0
Low	2	6.31 (0.05) [ab]	6.30 (0.07) [ab]	0.01
Low	3	6.24 (0.07) [bc]	6.24 (0.13) [ab]	0
Low	4	6.28 (0.03) [ab]	6.20 (0.10) [ab]	0.08
Low	5	6.15 (0.02) [dc]	5.78 (0.07) [c]	0.37*
Low	6	6.18 (0.02) [d]	5.97 (0.17) [bc]	0.21

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.8
Comparison of means (SD) for soil viable bacteria using RIM medium (Brim) after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between Brim after acidification and neutralization adjustments are calculated using logarithm transformed data, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively (paired t-test)

Metal	pH trt.	Brim (Log CFU) Neutralization	Brim (Log CFU) Acidification	Difference (neutral – acidi)
High	1	6.93 (0.11) [b]	6.93[†] (0.11) [a]	0
High	2	7.01 (0.07) [ab]	6.96 (0.19) [a]	0.05
High	3	7.06 (0.05) [ab]	6.90 (0.19) [a]	0.16
High	4	7.11 (0.03) [a]	6.92 (0.23) [a]	0.19
High	5	6.95 (0.08) [ab]	6.83 (0.16) [a]	0.12
Low	1	6.55 (0.02) [a]	6.55 (0.02) [a]	0
Low	2	6.66 (0.03) [a]	6.53 (0.07) [a]	0.12
Low	3	6.63 (0.01) [a]	6.40 (0.12) [ab]	0.23
Low	4	6.63 (0.03) [a]	6.36 (0.05) [b]	0.27*
Low	5	6.52 (0.04) [a]	6.19 (0.13) [c]	0.33
Low	6	6.24 (0.11) [b]	6.17 (0.08) [c]	0.07

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.9
Comparison of means (SD) for soil viable actinomycete (A) after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between the number of actinomycetes after acidification and neutralization adjustments are calculated using logarithm transformed data, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively (paired t-test)

Metal	pH trt.	A (Log CFU) Neutralization	A (Log CFU) Acidification	Difference (neutra – acidi)
High	1	7.03 (0.02) [a]	7.03[†] (0.02) [a]	0
High	2	6.99 (0.03) [ab]	6.92 (0.03) [ab]	0.07
High	3	6.98 (0.03) [ab]	6.95 (0.05) [ab]	0.03
High	4	6.94 (0.04) [bc]	6.82 (0.11) [bc]	0.12
High	5	6.88 (0.04) [c]	6.73 (0.07) [c]	0.15
Low	1	6.59 (0.04) [a]	6.59 (0.04) [a]	0
Low	2	6.63 (0.01) [a]	6.54 (0.06) [a]	0.09
Low	3	6.60 (0.03) [a]	6.55 (0.08) [a]	0.05
Low	4	6.61 (0.02) [a]	6.54 (0.07) [a]	0.07
Low	5	6.49 (0.05) [b]	6.41 (0.07) [b]	0.08
Low	6	6.38 (0.03) [c]	6.30 (0.11) [b]	0.08

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.10
Comparison of means (SD) for soil viable fungi (F) after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between the number of fungi after acidification and neutralization adjustments are calculated using logarithm transformed data, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively (paired t-test)

Metal	pH trt.	F (Log CFU) Neutralization	F (Log CFU) Acidification	Difference (neutra – acidi)
High	1	4.68 (0.12) [a]	4.68[†] (0.12) [ab]	0
High	2	4.60 (0.11) [a]	4.71 (0.09) [ab]	-0.11
High	3	4.67 (0.07) [a]	4.64 (0.09) [ab]	0.03
High	4	4.52 (0.06) [a]	4.59 (0.15) [b]	-0.07
High	5	4.51 (0.05) [a]	4.79 (0.14) [a]	-0.28
Low	1	4.43 (0.12) [b]	4.43 (0.12) [b]	0
Low	2	4.68 (0.07) [a]	4.45 (0.12) [ab]	0.23*
Low	3	4.67 (0.08) [a]	4.46 (0.21) [ab]	0.21
Low	4	4.60 (0.05) [ab]	4.48 (0.14) [ab]	0.12
Low	5	4.71 (0.05) [b]	4.66 (0.08) [a]	0.05
Low	6	4.47 (0.11) [c]	4.50 (0.02) [ab]	-0.03

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.11
Comparison of means (SD) for the most probable number (MPN) of indigenous *R. leguminosarum* bv. *trifolii* after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between the number of *rhizobium* after acidification and neutralization adjustments are calculated using logarithm transformed data, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively (paired t-test)

Metal	pH trt.	*Rhizobium* (Log MPN) Neutralization	*Rhizobium* (Log MPN) Acidification	Difference (neutra – acidi)
High	1	7.58 (0.21) [a]	7.58[†] (0.21) [a]	0
High	2	7.47 (0.36) [a]	6.70 (0.39) [ab]	0.77
High	3	7.04 (0.28) [a]	5.98 (0.54) [b]	1.06*
High	4	5.34 (0.64) [b]	3.42 (0.74) [c]	1.92*
High	5	2.56 (0.56) [c]	-0.07 (0.38) [d]	2.63**
Low	1	8.59 (0.25) [a]	8.59 (0.25) [a]	0
Low	2	8.83 (0.32) [a]	8.93 (0.60) [a]	-0.10
Low	3	8.44 (0.59) [ab]	8.39 (1.02) [a]	0.05
Low	4	7.10 (0.85) [b]	7.87 (0.84) [a]	-0.77
Low	5	2.20 (0.28) [c]	2.51 (0.52) [b]	-0.31
Low	6	0.74 (0.54) [c]	0 (0) [c]	0.74

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.12
Comparison of means (SD) for 0.1 M $Sr(NO_3)_2$ extractable Cd after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between the concentration of Cd after acidification and neutralization adjustments are analyzed using t-test, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively.

Metal	pH trt.	Cd ($mg\ kg^{-1}$) Neutralization	Cd ($mg\ kg^{-1}$) Acidification	Difference (neutral – acidi)
High	1	0.62 (0.01) [c]	0.62[†] (0.01) [e]	0
High	2	0.70 (0.01) [c]	0.81 (0.03) [d]	-0.11
High	3	0.68 (0.03) [c]	0.94 (0.04) [c]	-0.27*
High	4	1.13 (0.03) [b]	2.38 (0.09) [b]	-1.25**
High	5	1.56 (0.15) [a]	4.89 (0.16) [a]	-3.33***
Low	1	0.21 (0) [a]	0.21 (0) [d]	0
Low	2	0.21 (0) [a]	0.21 (0) [d]	0
Low	3	0.21 (0) [a]	0.21 (0) [d]	0
Low	4	0.21 (0) [a]	0.31 (0.01) [c]	-0.11**
Low	5	0.21 (0) [a]	0.95 (0.05) [b]	-0.74***
Low	6	0.22 (0.01) [a]	1.17 (0.12) [a]	-0.94**

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.13
Comparison of means (SD) for 0.1 M $Sr(NO_3)_2$ extractable Zn after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between the concentration of Zn after acidification and neutralization adjustments are analyzed using t-test, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively.

Metal	pH trt.	Zn (mg kg^{-1}) Neutralization	Zn (mg kg^{-1}) Acidification	Difference (neutra – acidi)
High	1	3.61 (0.08) ^{c}	3.61† (0.12) ^{e}	0
High	2	4.36 (0.11) ^{bc}	5.64 (0.11) ^{d}	-1.28*
High	3	4.19 (0.25) ^{bc}	7.07 (0.56) ^{c}	-2.88*
High	4	9.53 (0.31) ^{b}	32.0 (3.05) ^{b}	-22.4**
High	5	19.1 (2.61) ^{a}	97.0 (5.90) ^{a}	-77.9***
Low	1	0.30 (0) ^{c}	0.30 (0) ^{d}	0
Low	2	0.30 (0) ^{c}	0.31 (0.01) ^{d}	-0.01
Low	3	0.31 (0.01) ^{c}	0.34 (0.02) ^{d}	-0.03
Low	4	0.39 (0.05) ^{bc}	2.37 (0.15) ^{c}	-1.98**
Low	5	0.63 (0.08) ^{ab}	14.8 (0.63) ^{b}	-14.1***
Low	6	0.70 (0.14) ^{a}	18.6 (1.86) ^{a}	-17.9**

† Acidification values were adjusted by a ratio based on the control soil values.

Table 6.14
Comparison of means (SD) for 0.1 M $Sr(NO_3)_2$ extractable Al after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between the concentration of Al after acidification and neutralization adjustments are analyzed using t-test, "*","**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively.

Metal	pH trt.	Al (mg kg^{-1}) Neutralization	Al (mg kg^{-1}) Acidification	Difference (neutra – acidi)
High	1	0.39 (0.04) [a]	0.39[†] (0.06) [b]	0
High	2	0.44 (0.03) [a]	0.41 (0.08) [b]	0.03
High	3	0.48 (0.04) [a]	0.40 (0.07) [b]	0.08
High	4	0.41 (0.04) [a]	0.42 (0.07) [b]	-0.01
High	5	0.50 (0.08) [a]	0.59 (0.08) [a]	-0.08
Low	1	0.57 (0.05) [a]	0.57 (0.08) [c]	0
Low	2	0.46 (0.03) [ab]	0.50 (0.09) [c]	-0.03
Low	3	0.39 (0.03) [b]	0.50 (0.09) [c]	-0.11
Low	4	0.43 (0.03) [b]	0.62 (0.12) [c]	-0.19
Low	5	0.43 (0.02) [b]	2.21 (0.52) [b]	-1.78*
Low	6	0.47 (0.04) [ab]	13.6 (5.00) [a]	-13.1

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.15
Comparison of means (SD) for 0.1 M $Sr(NO_3)_2$ extractable Mn after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between the concentration of Mn after acidification and neutralization adjustments are analyzed using t-test, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively.

Metal	pH trt.	Mn ($mg\ kg^{-1}$) Neutralization	Mn ($mg\ kg^{-1}$) Acidification	Difference (neutra – acidi)
High	1	3.11 (0.18) [b]	3.11[†] (0.28) [e]	0
High	2	3.37 (0.11) [b]	4.47 (0.35) [d]	-1.10
High	3	3.38 (0.15) [b]	5.17 (0.65) [c]	-1.78
High	4	5.06 (0.38) [a]	12.0 (0.68) [b]	-6.97**
High	5	6.47 (0.53) [a]	22.7 (1.08) [a]	-21.6*
Low	1	0.57 (0.03) [c]	0.57 (0.04) [f]	0
Low	2	1.48 (0.04) [b]	1.41 (0.16) [e]	0.07
Low	3	1.75 (0.09) [b]	2.73 (0.40) [d]	-0.98
Low	4	1.96 (0.17) [b]	6.66 (0.77) [c]	-4.70*
Low	5	3.50 (0.15) [a]	16.0 (2.68) [b]	-12.5*
Low	6	3.04 (0.40) [a]	24.6 (4.32) [a]	-21.6*

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.16
Comparison of means (SD) for 0.1 M $Sr(NO_3)_2$ extractable Ca after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between the concentration of Ca after acidification and neutralization adjustments are analyzed using t-test, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively.

Metal	pH trt.	Ca ($mg\ kg^{-1}$) Neutralization	Ca ($mg\ kg^{-1}$) Acidification	Difference (neutra – acidi)
High	1	2210 (25.1) [a]	2210[†] (38.2) [a]	0
High	2	2137 (15.3) [a]	2103 (56.6) [a]	34.5
High	3	2229 (24.9) [a]	2036 (48.2) [a]	192
High	4	2176 (23.2) [a]	1769 (76.9) [b]	407*
High	5	2112 (68.1) [a]	1359 (98.4) [c]	753***
Low	1	2400 (23.7) [a]	2400 (35.9) [a]	0
Low	2	2166 (18.0) [b]	2067 (72.8) [ab]	99.1
Low	3	2132 (43.7) [b]	1844 (60.0) [bc]	288*
Low	4	2047 (24.3) [b]	1496 (38.7) [c]	552**
Low	5	1816 (20.3) [c]	1131 (65.7) [d]	686**
Low	6	1818 (46.8) [c]	947(172) [d]	870*

[†] Acidification values were adjusted by a ratio based on the control soil values.

Table 6.17
Comparison of means (SD) for 0.1 M $Sr(NO_3)_2$ extractable Mg after acidification (Acidi) and neutralization (Neutra) adjustments. Values with different superscript letters for the same soil within each column are significantly different with respect to pH treatments (LSD t-test, $p < 0.05$). Difference between the concentration of Mg after acidification and neutralization adjustments are analyzed using t-test, "*", "**", and "***" indicate the significance at 0.05, 0.01, and 0.001 probability levels, respectively.

Metal	pH trt.	Mg ($mg\ kg^{-1}$) Neutralization	Mg ($mg\ kg^{-1}$) Acidification	Difference (neutra – acidi)
High	1	433 (4.53) [a]	433[†] (6.87) [a]	0
High	2	421 (4.18) [a]	462 (17.6) [a]	-40.9
High	3	391 (4.82) [b]	446 (13.9) [a]	-55.5*
High	4	292 (2.76) [c]	372 (13.6) [b]	-80.1**
High	5	200 (7.95) [d]	292 (40.9) [c]	-91.3
Low	1	103 (0.62) [b]	103 (0.93) [ab]	0
Low	2	118 (2.62) [ab]	103 (13.0) [ab]	15.5
Low	3	121 (1.93) [a]	116 (7.44) [a]	5.67
Low	4	113 (7.80) [ab]	134 (17.3) [a]	-21.8*
Low	5	81.4 (6.65) [c]	115 (22.9) [a]	-33.7
Low	6	43.9 (3.14) [d]	75.4(11.7) [b]	-31.6

[†] Acidification values were adjusted by a ratio based on the control soil values.

Chapter 7 Overall Conclusions

Reducing soil pH is a double-edged sword: on the one hand, reducing soil pH can increase plant metal uptake because more metals become easily available for plants ; on the other hand, when pH is reduced, soil biological activities are reduced and microbial communities are shifted, which could exert more pressure on an already vulnerable soil ecology system. What is the relationship of these two effects? How do they interact with each other? Is there a threshold of pH value to balance plant growth, metal uptake and a healthy soil ecology system? Is it justifiable to use lowering pH as a method to enhance phytoextraction? We have to find that point when satisfactory phytoextraction and a healthy soil ecosystem are balanced.

This research answered many of the above questions. For the high metal soil, best plant growth was at the lowest pH level while the highest metal concentration was observed at the second lowest pH treatment. For low metal soil, due to low pH induced Al and Mn toxicity, both plant growth and metal uptake was the best at intermediate pH levels. Reducing pH significantly lowered soil biological activities and shifted the community structure at different levels. Ranging from 7.27 to 4.74, the threshold pH values were 6.1 and 5.3 for low and high metal soils, respectively. Above these values, most of the soil biological activities and all tested microbial populations returned to background levels within a short period. While below these values, the soil biological activities and microbial populations could be permanently damaged or would take a significantly longer time to recover.

These results show that reducing soil pH too far does not necessarily always lead to better plant growth and higher metal uptake, but may worsen the soil ecological system.

In other words, the tradeoff between metal uptake and soil ecological system is not always balanced when soil pH is continuously reduced. Furthermore, because soil biological activities and microbial populations can be severely damaged when soil pH is reduced below a certain threshold, precaution must be exercised when we try to enhance phytoextraction by reducing soil pH. Fortunately, present research revealed that it is not necessary to reduce pH too much. Optimum plant growth and phytoextraction can be achived before pH being lowered to a value where possibly permanent ecologic damage would occur. Based on our results, the pH threshold can vary according to the soil type, mineral composition and other properties. There is no unique solution to all different kinds of soils.

It is worthwhile to note that by using sequential metal extraction, we are able to assess the redistribution of Cd and Zn among five fractions caused by reducing pH. Generally, the most soluble metal form was greatly increased with decreasing pH. Less soluble fractions had different degrees of mobilization under low pH. In addition, results indicated that *T. caerulescens* was able to differentially utilize Cd in all 5 fractions while only get access to Zn primarily from F1 and F2 pools. Continuous monitoring of soil solution equilibrium is needed to provide information on long-term dynamics of metal distribution before multiple-year-phytoextraction being complete.

Overall, this research demonstrated that reducing pH is effective to enhance phytoextraction and is acceptable from ecologic point of view as long as soil pH maintained at certain reasonable levels.

Appendix 1. Formula of RIM medium

				LITER^{-1}
content	STOCK g l	Stock Prep.	g	ml
$Ca(NO_3)_2$	75.1	Autoclave		10
$MgSO_4$	246.5	Autoclave		1
ACES			9.11	
NaOH	400			2.5
Bacto Agar			15	
pH 6.7 - 6.9				
Add H_2O				to 1000
AUTOCLAVE	Mixture	Cool to 55^{o}C		
KH_2PO4	136.1	pH 7; Autoclave		1
Dextrose		1 Autoclave		1
Amino Acid Mix:				
Glycine	6.7			
Alanine	6.7			
Valine	6.7			
Leucine	6.7			
Isoleucine	6.7			
Serine	6.7			
Methionine	6.7			
Phenylalanine	6.7			
Tryptophan	6.7			
Glutamine	6.7			
Glutamic Acid	6.7			
Histidine	6.7			
Arginine	6.7			
Lysine	6.7			
Tyrosine	6.7	NaOH;Autoclave		1
Vitamin Mix:		Filter		1
Biotin	0.1			
Niacinamide	0.35			
Thiamine•2HCl	0.3			
ANTIBIOTICS	inhibit fungi			
Cycloheximide inhibit fungi (Rhizoctonia)	100 ug			1
Nystatin Inhibit fungi & action.	10	Water/Methanol 2:1		

Appendix 2. Formula of R2A medium

Content	g L^{-1}
Yeast extract	0.5
Bacto proteose peptone	0.5
Bacto casamino, acids	0.5
Bacto dextrose	0.5
Soluble starch	0.5
Sodium pyruvate	0.3
Potassium phosphate, dibasic	0.3
Magnesium sulfate	0.05
Agar	15
Cycloheximide/dissolve in ethanol	100mg

Appendix 3. Formula of Martin's medium

Content	g L^{-1}
Dextrose/Glucose	10
Peptone	5
Rose Bengal	0.033
Streptomycin	0.03
KH_2PO_4	0.5
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.5
Agar	15

Appendix 4. Formula of Starch Casein medium

Content	g L^{-1}
Dextrose/Glucose	10
Peptone	5
Rose Bengal	0.033
Streptomycin	0.03
KH_2PO_4	0.5
K_2HPO_4	0.5
$MgSO_4 \cdot 7H_2O$	0.5
Agar	15

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